BIOLOGICAL TREATMENT AND RECYCLING OF TEXTILE PROCESSING EFFLUENTS

by

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AUTHOR 'S DECLARATION

This thesis is entirely my own work and has at no time been submitted for another degree.

I certify that this statement is correct

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ABSTRACT

In the present work, a mixed culture of *Pseudomonas* spp. capable of decolourising a range of selected textile dyes was isolated and used to develop a continuous culture system for the treatment of textile dye effluents. The bioprocess was optimised using biomass growth supports. The presence of a carbon source such as soluble wheat starch (0.2 % w/v) in dye solution media and effluent samples enhanced decolourisation. A polymer support (polyurethane foam) was used for immobilisation of the bacteria in the laboratory-scale bioreactor, and helped create an integrated anaerobic / aerobic condition within the foam matrix and promote degradation of azo dyes and organic compounds. The system showed high levels of decolourisation up to 98 % over 12 days of continuous operation. However, toxicity levels of dye samples increased up to 65 % after anaerobic biotreatment, due to the formation of toxic aromatic amines. The continuous culture bioprocess was also combined with membrane filtration technology to improve effluent treatment. Decolourised, filtered effluents showed great reduction in COD, BOD_5 and toxicity levels, and were found suitable for re-use in dyeing processes. Dyed cotton fabrics did not show any significant difference with those dyed using normal supply water.

These studies show great potential for improvement of an existing industrial effluent treatment plant through the use of biomass growth supports and the combination of membrane technology. Considerable savings are foreseeable through the implementation of the process, provided effluent recycling within the textile factory is successful.

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CHAPTER 1

INTRODUCTION

The research was part of a European project, involving the collaboration of five partners from EU countries and one associated country. The objective of the project was to study and develop biotechnical treatments and recycling of textile processing effluents (BIOEFFTEX – *GRD1-1999-10435*).

Currently, the European textile industry faces harsh competition from Far-East countries, where labour costs are low. The development of new technologies for waste water treatment will create more efficient and cost-effective processes and could improve the economical situation if successfully applied. Such technological progress could also be exploited by other industries such as paper, plastic, automobile, dyestuff producing and pharmaceuticals.

The existing sewage treatment methods are said to be "end of pipe" techniques from an ecological and economical point of view as they are not ideal for treating the effluents to the high quality required for discharged water. Textile processing effluents are complex, composed of undefined substances resulting from various dyeing and process steps and often require expensive multi-stage treatments. The need to improve waste water treatment is evident since they constitute an important part of the total costs of a textile company.

Research towards environmentally friendlier process alternatives is essential with the strict legislations on discharge limits and toxic impact of wastes on the environment. The development and optimisation of innovative processes for effluent treatment would lead to environmental benefits.

In order to achieve these goals, some research aspects were drawn through the project and involved:

- The use of defined recyclable enzymes and or microorganisms for degradation of dyestuffs in effluents, and hence removal of colour and high organic load from the effluent
- Lower amount of biomass in smaller reactor volumes
- Faster reaction on effluents
- Combination of biotechnical and or physical methods (e.g. membrane technology)
- Water reuse for textile dyeing and finishing without impact on quality of end-products
- Development of appropriate industrial equipment

Chapter 2 gives an insight of the current conditions on textile effluent treatments used in the textile industry and the legislations dealing with industrial effluent discharges, with some information from the published literature on the toxicity of dye-degradation products giving rise to health and safety concerns.

The research was carried out in collaboration with a local textile company, Quantum Clothing Ltd Stevensons, who provided samples of their effluents for analyses.

The design and development of a low cost biotechnical effluent treatment system that brings environmentally sound solutions to the problem of colour in textile effluents was important to the research project. In order to develop such a treatment system, a microbial culture capable of decolourising a wide range of textile dyes was isolated and tested. The determination of optimum batch conditions for colour removal is described in this thesis. The variables investigated were the carbon sources used in the media preparations and the biomass supports for immobilisation of bacteria. The treatment process was finally set up to assess the efficiency of the new system.

Publications

The work presented in this thesis was also partially included in the following papers:

Do T., Shen J., Cawood G. and Jenkins R.O. (2002) Biotreatment of Textile Effluent Using *Pseudomonas* spp Immobilised on Polymer Supports. In: Hardin I., Akin D., Wilson S. (Eds), *Advances in Biotechnology for Textile Processing.* The University of Georgia, Athens, USA, 35-45.

Do T., Jenkins R., Shen J. and Cawood G. (2002) Integrated Bio-treatment, *GB Patent, Application Nº. 0222569.6.*

Do T., Shen J., Cawood G. and Jenkins R. (2002) Improvement of textile effluent treatment for colour removal using immobilised bacteria on polymer support. Poster at the *151st Ordinary Meeting of Society for General Microbiology, FB03,* 16-20 September 2002, University of Loughborough, U.K.

CHAPTER 2

LITERATURE REVIEW

2.1 Properties of water used in and discharged by the textile industry

2.1.1 Sources of water and characterisation of textile effluent

There is unequal distribution of water within the UK. The lowest rainfall occurs in Southern and Eastern England, which also have the highest population density. The UK Government's waste policy emphasises that water in the UK cannot be treated as an unlimited resource; hence there is a growing interest in reducing the demand for water by industry. According to Holt *et al.* (2000), companies are able to reduce water consumption by approximately 30 %. By using water recycling technology, the industry can make significant financial savings. As demand for water in the UK is continuously increasing with the increase of population density, companies are encouraged to make more efficient use of the resources in order to reduce water consumption and waste production.

Textile industries consume huge quantities of water. The sources of water supply are usually: river, borehole and town mains. Some water sources such as town water are often unsuitable for textile processes such as dyeing because they contain suspended matter and soluble calcium and magnesium salts. Hence filtration and softening are often required (Little, 1975). Table 2.1 shows the acceptable limits of some substances present in water for use in textile processing. The criteria for water used in textile processing are, according to Little (1975), no suspended solids or staining substances present in the water, no excess of acid/alkali, no substances such as iron, manganese, calcium, magnesium salts and heavy metals, no substances that are corrosive to tanks and pipelines and no substances giving foams or unpleasant odours.

Sampling and analysis of water supply give useful information for controlling water quality for textile processing. In order to find suitable effluent treatment, the nature of the effluent components should be determined by chemical and biological analysis. Textile finishing effluent can vary greatly from plant to plant. Its nature depends on the type of production and the type of textile auxiliary agents and dyes employed and other factors (Schulze-Rettmer *et al.*, 2000). Chemicals are used for textile wet processing, including desizing, scouring, bleaching, dyeing, printing and finishing. A range of inorganic compounds, polymers and organic products are used during textile processing. This implies that the composition of textile effluent is very varied.

Table 2.1 Acceptable limits of substances present in water for textile uses (acceptable water quality for textile use) (Little, 1975)

Quality or Substance	Acceptable Limits
Turbidity (mg/L)	< 5
Suspended solids (mg/L)	< 5
Colour (unit, Hazen)	< 10
pH	7-9
Acidity/alkalinity (mg/L)	< 100 as $CaCO_3$
Hardness (mg/L)	< 70 as $CaCO_3$
Iron (mg/L)	< 0.3
Manganese (mg/L)	< 0.05
Copper (mg/L)	< 0.01
Lead or heavy metals (mg/L)	< 0.01
Aluminium (mg/L)	< 0.25
Silica (mg/L)	< 10
Sulfate (mg/L)	< 250
Sulfide (mg/L)	< 1
Chloride (mg/L)	< 250
Phosphate (mg/L)	No limit
Dissolved oxygen (mg/L)	No limit
Carbon dioxide (mg/L)	< 50
Nitrite (mg/L)	< 0.5
Chlorine (mg/L)	< 0.1
Ammonia (mg/L)	< 0.5
Oil, grease, fat, soap (mg/L)	< 1
Fluorescent brightening agents (mg/L)	< 0.2
Total solids (mg/L)	< 500

Table 2.2 shows an example of the analysis of effluent from a mill processing cotton and gives typical values for a textile effluent sample. Conductivity is measured in MicroSiemens per metre and is a measure of the water's ability to carry an electric current, which is usually caused by the presence of salts such as NaCl. The chemical oxygen demand (COD) corresponds to the amount (mg/L) of oxygen consumed during oxidation of a test substance with hot, acidic dichromate; it provides a measure of the amount of oxidizable matter present, expressed as mg oxygen consumed per mg of test substance. The biochemical oxygen demand (BOD_5) is the amount (mg/L) of oxygen consumed by microorganisms when metabolising a test substance, usually under prescribed conditions; expressed as mg oxygen uptake per mg test substance (Painter, 1995), hence BOD_5 measures the organic matter present in the water sample, which is biologically decomposable within 5 days.

Table 2.2 Analysis of effluent from a cotton-processing mill (from Schulze-Rettmer *et al.*, 2000)

Parameter	Effluent
pH value	10
Conductivity µS/m	1000
Temperature °C	28
COD value mg/L	2000
BOD_5 value mg/L	700
Total Kjeldahl Nitrogen (N) mg/L	25
Total phosphate (P) mg/L	4
Visual colouration	intense

2.1.2 Colour pollution in effluent

In the textile dyeing industry, 40 - 65L of wastewater is generated per kg cloth produced (Manu *et al.*, 2002). According to Ince *et al.* (1999), 1000 mg/L of dye is present in a typical dyebath. Table 2.3 shows the estimated degree of fixation of dyes to fibres and the percentage loss of dyes going into the effluent.

Reactive and sulfur dyes show the least dye-fixation and consequently a high percentage of the dyes are lost into effluent.

The presence of dyes and dye degradation products in waters causes problems such as acute and, or chronic effects on exposed organisms (toxicity), aesthetic problems, and environmental effects (absorption and reflection of sunlight in water), which interfere with bacterial growth and affect the food chain (Slokar *et al.*, 1998). Colour in textile dyehouse effluents is therefore of great concern to the industry and treatment to remove colour is essential before discharge to rivers. Effective treatment of textile effluent is difficult because as mentioned earlier it contains various types of chemicals, including residual dyes with complex structures and other pollutants such as salts, surfactants, nitrates, metal ions, dispersants, levelling agents, acids and alkali. Moreover, the composition of dyeing effluent varies with the textile produced. A single dyeing operation can use a number of dyes from different chemical classes resulting in a very mixed wastewater (O'Neill *et al.*, 1999).

Table 2.3 Estimated degree of fixation for different dye-fibre combinations and loss to effluent (Easton, 1995).

Dye class	Fibre	Degree of fixation (%)	Loss to effluent (%)
Acid	polyamide	80-95	5-20
Basic	Acrylic	95-100	0-5
Direct	Cellulose	70-95	5-30
Disperse	Polyester	90-100	0-10
Metal-complex	Wool	90-98	2-10
Reactive	Cellulose	50-90	10-50
Sulfur	Cellulose	60-90	10-40
Vat	Cellulose	80-95	5-20

Pollution in treated dyehouse effluents is mainly due to the presence of toxic dyes that are resistant to microbial degradation (Peralta-Zamora *et al.*, 1998). Hence the discharged effluents may still have an effect on fauna and flora. As high levels of other inorganic and organic materials might also be discharged into the environment, the wastewater needs to be treated, not only for colour removal but also for the removal of organic pollutants. Strong colour can reduce

light penetration, thus affecting the growth of plants and impacting on invertebrates and other forms of wildlife. There is a remarkably wide variety of colorants with different chemical and physical properties and it is not surprising that they also show toxicological properties.

High quality dyes are found to be more difficult to treat or degrade. Modern textile dyes are required to have high degree of chemical and photolytic stability in order to maintain their structure and colour (Easton, 1995). The colour fastness, stability and resistance of dyes to degradation have made colour removal from textile wastewaters difficult (Boe *et al.*, 1993). The synthesis of dyes is thoroughly controlled as dyes containing carcinogenic aromatic amines are strictly forbidden by the Ecological and Toxicological Association of the Dyestuffs (ETAD) (see Section 2.2.1)

2.1.3 Textile dyes

Over 10,000 commercially available dyes exist with over $7x10^5$ metric tons of dyestuff produced annually (Meyer, 1981; Zollinger, 1987). Dyes can be classified by their chemical structures or application method (Kirk-Othmer, 1993), but they all absorb light in the visible region (Table 2.4). As mentioned earlier, dyes are difficult to treat because of their synthetic origin and their complex aromatic molecular structures; they are usually constructed to resist biological attack, light, heat and oxidation. That makes them more stable and hence difficult to biodegrade.

Each type of dye is different in the way it reacts in a dyeing bath and in effluent. Water insoluble pigments are usually completely extracted from the effluent during treatment. Residual effluent colouration is mainly due to dyeing processes using reactive dyes, which have a low degree of fixation to the fibre. Up to 40 % of initial dyes remain unfixed and end up in dye-bath effluent (Shah, 1998) (See Table 2.3). A typical concentration of 800 mg/L of hydrolysed dye could remain in the bath. Since they constitute a large part of current dye

production, they pose the biggest problem in effluent coloration (Schulze-Rettmer *et al.*, 2000).

Table 2.4 Application classes of dyes and their chemical type (Kirk-Othmer, 1993).

Class	Method of application	Chemical types
Acid	From neutral to acidic dyebaths	Azo including premetallised anthraquinone, triphenylmethane, azine, xanthene, nitro and nitroso
Azoic components	Fibre impregnated with coupling component and treated with a solution of stabilised diazonium salt	Azo
Basic	Applied from acidic dyebaths	Diazacarboncyanine, cyanine, hemicyanine, azo, azine, xanthene, acridine, oxazine and anthraquinone
Direct	Applied from neutral or slightly alkaline baths containing additional electrolyte	Azo, phthalocyanine, stilbene and oxazine
Disperse	Fine aqueous dispersions often applied by high-temperature pressure or lower temperature carrier methods	Azo, anthraquinone, styryl, nitro and benzodifuranone
Fluorescent brighteners	From solution, dispersion or suspension in a mass	Stilbene, pyrazoles, coumarin and naphthalimides
Food, drug and cosmetic	N/A	Azo, anthraquinone, carotenoid and triarylmethane
Mordant	Applied in conjunction with chelating Cr salts	Azo and anthraquinone
Natural	Applied as mordant, vat, solvent or direct and acid dyes	Anthraquinone, flavonols, flavones, indigoids, chroman
Oxidation bases	Aromatic amines and phenols oxidized on the substrate	Aniline black and indeterminate structures
Pigments	Printing on the fibre with resin binder or dispersion in the mass	Azo, basic, phthalocyanine, quinacridone and indigoid
Reactive	Reactive site on dye reacts with functional group on fibre to bind dye covalently under influence of heat and pH (alkaline)	Azo, anthraquinone, phthalocyanine, formazan, oxazine and basic
Solvent	Dissolution in the substrate	Azo, triphenylmethane, anthraquinone and phthalocyanine
Sulfur	Aromatic substrate vatted with sodium sulfide and re-oxidized to insoluble sulfur-containing products on fibre	Indeterminate structures
Vat	Water-insoluble dyes solubilised by reducing with sodium hydrosulfite, then exhausted on fibre and re-oxidized	Anthraquinone (including polycyclic quinones) and indigoids

The cotton industry is strongly associated with coloured effluent, unlike the wool industry where up to 98 % of the dye is absorbed by the wool resulting in less coloured effluent (O'Neill *et al.*, 1999). Basic dyes have high brilliance, hence higher colour intensity, which make them more difficult to decolourise. Metal-based complex dyes (e.g. chromium-based dyes) can lead to the release of chromium, which is carcinogenic, into the environment. Some disperse dyes have been shown to have a tendency to bioaccumulate (Anliker *et al.*, 1981; Baughman *et al.*, 1988). Indeed, heavy-metal ions from textile effluents have been reported at high concentration in algae and higher plants exposed to such effluents (Srivastava *et al.*, 1991).

Azo dyes are extensively used for dyeing of cotton and constitute about 60 to 70 % of total dyes produced. They are characterised by -N=N- bonds (most common chromophore of reactive dyes) (O'Neill *et al.*, 1999). Commercial dyes are mainly prepared by diazotizing aromatic primary amines and coupling the diazonium salts with phenols or aromatic amines with free *ortho* and, or *para* positions having high electron densities, or with other compounds having reactive positions. The dyes can contain more than one azo linkage. In azo dye manufacture, some intermediates (e.g. benzidine, 2-naphthylamine and other aromatic amines) are carcinogenic or toxic (Levine, 1991). This is thoroughly controlled by the ETAD (see Section 2.2.1), which states that it is strictly forbidden to synthesise dyes containing carcinogenic aromatic amines.

Quantitative and qualitative characteristics of azo dye colours depend on the number and position of azo groups, the nature and position of substituents, e.g. halo, alkyl, hydroxyl, carboxyl, nitro, and especially sulfonate, and on the nature of the aromatic nucleus, e.g. benzene, naphthalene, pyrazolone. The higher the number of azo linkages, the more colourfast the dyes are, although compounds with more than 2 linkages are considered less attractive (Levine, 1991). Azo and reactive dyes are electron deficient in nature, hence less susceptible to oxidative catabolism (Knackmuss, 1996). They are also hydrophilic in nature and hence can pass through the conventional aerobic process untreated. Some azo dyes used on textiles for body-contact end-uses have been withdrawn from

the textile industry by the government in Germany because they produce carcinogenic amines as degradation products. However, many acid and direct dyes, which may liberate amines such as benzidine and o-toluidine, are still in use (O'Neill *et al.*, 1999).

According to Harmer *et al.* (1992), dyes are usually not cytotoxic, mutagenic or carcinogenic, but after anaerobic digestion, they form aromatic amines that may be toxic and possess these characteristics. It is believed that azo dyes are decolourised by reduction of the azo bond with which the colour is associated, via anaerobic degradation through the action of non-specific enzymes (Boe *et al.*, 1993; Zaoyan *et al.*, 1992). Dyes are reduced when acting as electron acceptors for the microbial electron transport chain, and a source of labile carbon is therefore required to sustain the bacteria (Carliell *et al.*, 1996).

The sources of water pollution in the textile industry are desizing, scouring, bleaching and dyeing processes. Textile wastewaters can be characterised by their highly visible colour, COD, alkaline pH and Total Solids (TS). Due to the shortage of water sources and more stringent effluent standards imposed by the regulatory authorities more efficient and economical treatment technologies are required (Manu *et al.*, 2002).

2.2 Legislation on effluent disposal

2.2.1 Regulations on water and industrial effluent

The Ecological and Toxicological Association of the Dyestuffs manufacturing industry (ETAD) was formed in 1974 and aims to minimize environmental damage, protect users and consumers and to co-operate fully with the government and public concerns over the toxicological impact of their products (Anliker, 1979). The 1974 Water Pollution Act stated that the control of sewers and discharges should be taken over by ten regional water authorities. Following the Water Pollution Act, the Water Industry Act (1989) was brought in and helped to establish the National Rivers Authority (NRA) and privatised the

water services in England and Wales. This act made the polluter solely responsible for the costs of the required effluent control and treatment. The NRA was responsible for the management of the water environment. Its responsibilities extended to all "controlled" waters. The duties of NRA were extensive (Cooper, 1995):

- Maintenance of water quality and pollution control
- Management of water resources and licensing of abstractions
- Construction and maintenance of flood defences
- Maintenance, improvement and development of fisheries
- Promotion of conservation and recreation in the aquatic environment

The NRA had to balance these demands as well as provide for water supplies and means of effluent disposal.

The Water Industry Act was updated in 1991 and incorporated the specified duties of sewerage undertakers, the local authorities responsible for water supply, and increased the controls for discharge to the sewerage system and the quality of water supplies.

In the UK, these matters are now regulated by the Environment Agency (EA) for England and Wales and the Scottish Environment Protection Agency (SEPA) (Willmott *et al.*, 1998), which took over the NRA and the Water Authorities. The EA was established by the Environment Act, 1995. Its role is to regulate waste management through a system of licences and give advice on management methods including waste minimisation. The EA also monitors transport of waste, especially potentially hazardous waste. For a discharger to release effluent to sewers, consent from the water company is required, which in turn needs authorization from the EA (Cooper, 1995).

Environmental policy in the UK since September 1997 has stated that zero synthetic chemicals should be released into the aquatic environment. Enforcement of this law continues to ensure that textile industries treat their dye-containing effluent to the required standard (Robinson *et al.*, 2001).

Water Resources Act 1991

This act is in place to control discharge to watercourse in order to avoid pollution. The responsibility for controlling discharges lies with the Environment Agency (EA).

According to this act, it is an offence to cause or knowingly permit:

- Any poisonous, noxious or polluting substance to enter any controlled waters (coastal, lakes, ponds and ground waters)
- Any matter other than trade or sewage effluent to be discharged from a sewer in contravention of a relevant prohibition
- Any trade or sewage effluent to be discharged into controlled waters or, through a pipe, into the sea
- Any trade or sewage effluent to be discharged onto land or into a pond in contravention of a relevant prohibition
- Any matter to enter inland waters so as to cause or aggravate pollution by impeding the flow

With the agreement of the sewage undertaker, companies are allowed to discharge their effluents to the public sewer. Nevertheless, many small companies are unaware of the requirement of obtaining permission from the local water company to discharge to sewers.

2.2.2 Colour discharge consents

The EA decided that the best way to measure colour and set standards is by absorbance values over a range of wavelengths. To test rivers or effluent, samples are taken and filtered through a 0.45 μm filter, and absorbency in a 1cm cell is measured between 400 and 700 nm.

Colour in textile dyehouse effluents is of great concern to the textile industry. Treatment to remove colour is required prior to effluent discharge to rivers.

Abnormal colouration of surface waters affects the public and authorities and is due to the presence of residual dyestuff in waste streams (Slokar *et al.*, 1998; Banat *et al.*, 1996). The eye can detect concentrations of 0.005 mg/L of reactive dye in water (Pierce, 1994). Dye concentrations above the visual detection threshold are not permitted and according to O'Neill *et al.* (1999), consent levels for the discharge of colour into receiving waters are applied for aesthetic reasons, and not for prevention of toxicity. It is believed that dyes exhibit low toxicity to mammals and aquatic organisms (Churchley, 1998). New regulations have put pressure on water companies in the UK to reduce the amount of colour in sewage effluent. In 1992 in the UK, colour was the subject of more than 500 complaints, mostly from the Severn Trent region. (Pierce, 1994; O'Neill *et al.*, 1999).

In the UK, the EA determines what may or may not be discharged to river water. The EA sets consents for discharges, and assesses the proposed concentration of parameters against an Environmental Quality Standard (EQS), which shows the concentration that should not be exceeded downstream of the discharge (O'Neill *et al.*, 1999).

The permitted colour of the discharge is calculated as (Environment Agency, 1998):

$$Cd = \frac{[(Cs \times Ft) - (Cu \times Fu)]}{Fd} \qquad (1)$$

Where:

Cd Colour limit for discharge (absorbance nm^{-1})

Cs Colour standard for the watercourse (absorbance nm^{-1})

Cu Natural colour of watercourse upstream of discharge (absorbance nm^{-1})

Fd Mean discharge flow ($Mdm^3.day^{-1}$)

Ft Total river flow downstream of discharge ($Mdm^3.day^{-1}$)

Fu 95% excess river flow upstream of discharge ($Mdm^3.day^{-1}$)

The equation (1) takes into account the natural colour of the river; the excess colour from the sample is calculated and assessed to determine if it is within the range of acceptance.

2.2.3 The discharge of industrial trade effluent

Trade effluent refers to any liquid either with or without suspended solid particles, which is partly or completely produced in the course of trade or industry carried out on a trade premises. It is important to note that certain discharges to sewers are prohibited and might result in prosecution. Responsibility for discharge to sewers is in the hands of the local water authority.

The sewage undertaker agrees consent limits with the discharger depending on the volume and concentration of the effluent. These consents depend on the particular discharger and to formulate acceptable standards, the water authority asks the discharger to fill in an application called "Trade Effluent Notice" containing all the required information on the effluent to be discharged (e.g. nature, quantity and composition of the effluent). After assessment of the application, a decision is made about the consents, which the discharger will have to accept.

The reason why there is a request for a Trade Effluent Notice is mainly to ensure the security of the sewage system and the staff operating it (no damage to be caused by the Trade Effluent). It also aims at making sure that the effluent can be properly treated to prevent toxic substances entering the environment, by providing detailed information about its amount and nature. A consent document will be given when the application is accepted. It will contain the limits decided by the discharger and the water authority.

Cost of disposal

According to Moran (1995) who performed a recent survey for the Textile Finishers Association, around 80 % of textile finishers discharge their effluent to sewers, and pay local water industries to treat and dispose of it. Once under the charge of the water industry, it is subjected to standard sewage treatment and subsequently discharged to river (Moran *et al.*, 1997). Charges are made for the cost of services provided for discharge of effluents to the sewers.

The cost is based on the "Mogden formula". The reception, conveyance, treatment and disposal of effluent are taken into account in the calculation when charging the dischargers.

Calculation of the costs also includes the volume and quality of the discharged effluent (EA, 1999).

The Mogden formula:

$$C = R + V + \frac{(Ot)B}{(Os)} + \frac{(St)S}{(Ss)} \qquad (2)$$

Where:

R The cost of conveying waste into the sewage works (cost.m^{-3}.$year^{-1}$)

V The average cost of primary treatment at treatment works (cost.m^{-3}.$year^{-1}$)

Ot The Chemical Oxygen Demand (COD) of the discharge (mg/L after 1-hour quiescent settlement)

Os The average COD of all effluents received at the treatment works (mg/L after 1 hour quiescent settlement)

St The Total Suspended Solids (TSS) (mg/L)

Ss The average TSS of all effluent received at the treatment works (mg/L)

B The cost of biological oxidation treatment of settled sewage (unit cost.m^{-3})

S The cost of treatment and disposal of primary sludge (unit cost.m^{-3})

C Total charge for trade effluent (unit cost.m^{-3})

The factors B and S in equation (2) represent the cost of treating average COD and suspended solids levels in one m^3 of effluent in that region.

Dischargers who decide to discharge their effluent straight into watercourses must obtain consent from the Environment Agency. Usually, they are expected to have a pre-treatment plant at their dyehouse. The consents are normally stricter than those set by water authorities as the treated effluent will be directly discharged into the local watercourses. The discharger in this case is also monitored more regularly than those discharging to sewers. Charges (applied to discharges into local waters) are decided by the Environmental Agency under the Water Resources Act 1991. The payment takes account of the application fee, costs for processing the application, sampling, inspecting and monitoring the impact of the discharge, laboratory services, reviewing the consent and administration involved during the process.

According to Fig. 2.1, most of the water authorities seem to have increased water charges over two years from 1997 to 1999.

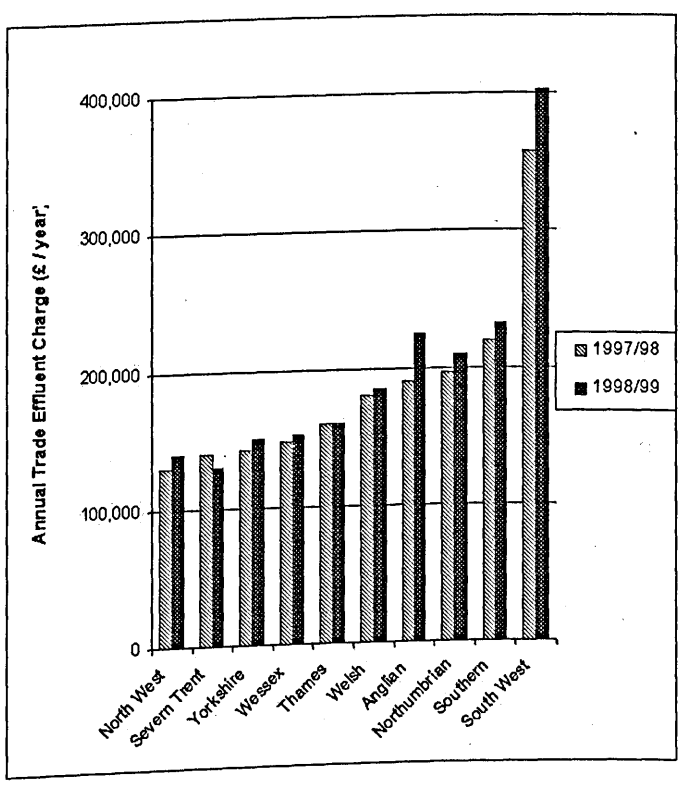


Figure 2.1 Water Plc charges in England & Wales in 1997/98 and 1998/99 (BMB Initiative - DTI, 1998).

Checks on the effluent quality are performed regularly in order to assess if it conforms to regulations. Tests consist of physical, chemical and biological tests, e.g. COD, BOD, pH, suspended solids, colour analysis and temperature. The monitoring of water and effluent quality is carried out regularly by the dischargers, and by the sewage works of the Environment Agency. This involves high costs for the dischargers who often decide not to carry out these tests.

The 1990 Environmental Protection Act (EPA) fines those who discharge without consent, but also those who have acquired permission and whose effluent does not conform to the standards agreed by the sewage works or the EA. In that case, the discharger risks to be blacklisted by the EA for discharging unsuitable effluent into watercourses. Similar action is taken on the sewage works if it is found guilty of receiving and processing unsuitable effluent.

The NRA is responsible for controlling and monitoring discharges into rivers, they deal with consents and enforcement of regulation in cooperation with the EA. The water authorities control discharges under the Water Industry Act 1991. They take care of water, sewage treatment, and river water quality and water distribution. They are themselves under the control of the EA. It is important to note that legislation on trade effluent has not always been efficient, the introduction of the Water Industry Act 1989 and the consequent privatisation of water services improved considerably the control of effluent discharges when they were placed under the control of ten regional water authorities in England & Wales (Fig. 2.1). Water authorities have put increasing pressure on the dischargers to improve the quality of their effluent before it reaches the sewage works. Moreover, prices for treatment have also increased due to the introduction of new equipment and methods to ensure the conformity of the effluent to regulations. This financial pressure has pushed some companies to install their own treatment plant to discharge straight into watercourses. These companies are controlled by the EA under The Water Resources Act 1991.

The dyeing industry has always been under great pressure to comply with regulations, which constantly get stricter and increase charges in order to decrease the risk of environmental damage. In addition, the tightening of colour standards for dyehouse effluent makes it difficult for textile industries to afford treatment of their effluent.

2.3 Conventional textile effluent treatment processes

There are three main process categories used to treat dye effluent in the textile industry. These categories are chemical, physical and biological, including enzymatic treatments. There is a constant effort in research to try to find the most effective and economical methods of treatment.

2.3.1 Chemical methods

Chemical methods include oxidative processes, Fentons reagent, ozonation, photochemical and electrochemical destruction of dye molecules.

Oxidative processes are the most widely used chemical methods as they are simple applications (Robinson *et al.*, 2001). The most commonly used oxidizing agent is hydrogen peroxide (H_2O_2), which needs activation (e.g. by ultraviolet) in order to cleave the aromatic ring in the dye molecules (Raghavacharya, 1997). Alternatively, sodium hypochlorite (NaOCl) may be used to promote and accelerate the destruction of the azo bond by attacking the amino group of the dye molecule. The increase in decolourisation is related proportionally with the increase in Cl^- concentration, as well as aromatic amines. This method is not very popular because of the problem associated with the release of Cl^- and aromatic amines into the environment (Slokar *et al.*, 1998). This treatment is usually not applicable to disperse dyes.

The method of Fentons reagent involves the use of a trapped iron Fe (II) and hydrogen peroxide H_2O_2 and is effective in treating soluble and insoluble dyes (Pak *et al.*, 1999). It uses sorption or bonding to eliminate the dissolved dyes

from the effluent. One of the disadvantages of this method is the fact that it generates sludge due to flocculation occurring between the reagent and the dyes (Robinson *et al.*, 2001) and hence further method of disposal are required for the produced sludge. It is important to note that unlike anionic dyes (acid, direct, vat mordant and reactive dyes), cationic dyes do not coagulate and consequently will influence the settling quality of the sludge formed.

Oxidation using ozone allows the degradation of a variety of compounds such as phenols, pesticides, aromatic and chlorinated hydrocarbons (Lin *et al.*, 1993; Xu *et al.*, 1999) and was first used in the 1970s (Robinson *et al.*, 2001). This technique does not produce sludge nor toxic metabolites (Gahr *et al.*, 1994). It removes colour in effluent usually after a reasonably short period of time and considerably decreases the COD, making the effluent ready to be discharged into the environment (Xu *et al.*, 1999).

Ozone is usually used in its gaseous form and hence does not affect the volume of the treated effluent, but its half-life is relatively short (only 20 minutes) and can be shorter when in presence of effluent components such as dyes, salt, alkaline pH, which affect its stability. Continuous ozonation is therefore required and this makes the technique very expensive because of the high capital costs and high running costs of the continuous ozonation plant (Robinson *et al.*, 2001).

The principle of the photochemical method is the degradation of dyes into carbon dioxide and water (Yang *et al.*, 1998; Peralta-Zamora *et al.*, 1999) by using ultraviolet treatment in the presence of hydrogen peroxide which forms a high concentration of hydroxyl radicals, which cause degradation of organic materials. By-products might be generated during the process, e.g. halides, metals, inorganic acids, organic aldehydes and organic acids, depending on the initial materials. Advantages for using this method are that no sludge is produced and effluent odours are reduced.

An electrochemical destruction method was recently developed and is very advantageous as it requires almost no chemicals and does not produce sludge. The breakdown products are normally not toxic and can be safely discharged into watercourses. It should be noted that flow rate affects the dye removal yield and electricity costs are comparable to chemicals (Robinson *et al.*, 2001).

2.3.2 Physical treatments

Adsorption can be very efficient in removing pollutants and obtaining high quality effluent. Decolourisation by adsorption is influenced by physico-chemical factors such as dye/sorbent interaction, sorbent surface area, particle size, temperature and contact time (Kumar *et al.*, 1998). The ultimate fate of adsorbed pollutants has been incineration or landfill disposal. The latter represents a concentration and accumulation of the pollutants. With the increasingly stringent regulations on environmental safety, this solution to a successful effluent treatment has become unacceptable, because the contaminants are not destroyed, but rather concentrated and buried, and may cause further complications as they degrade. Although incineration should lead to complete oxidation of adsorbed pollutants, there is widespread concern over dangerous products of incomplete combustion (such as dioxins) and atmospheric pollution by oxides of nitrogen and sulfur.

Activated carbon is the most widely used method of adsorption (Nasser *et al.*, 1991). The efficiency of this method depends on the type of carbon used and the nature of the wastewater. Its regeneration is nevertheless a limiting factor as well as its high cost.

Other types of sorbents are used in wastewater treatment such as peat, wood chips, fly ash, coal and silica gel.

Peat has a particular cellular structure that makes it capable of adsorbing transition metals and polar organic materials from dye effluents. Unlike activated carbon, peat is cheaper and does not require activation (Poots *et al*,

1976a). Its greater surface area makes it also a better sorbent. Wood chips are good adsorbents, especially for acid dyes, but usually longer contact times are required for good decolourisation (Poots *et al.*, 1976b).

A mixture of fly ash and coal is used in industry with good adsorption rates. Silica gels are also good sorbents, very effective in adsorbing basic dyes. However, it easily binds with substances found in the air (e.g. dust, or gas), which makes it commercially unsuitable.

Other types of adsorbent materials include natural and synthetic clays. Many companies in the Midlands have successfully used hydrotalcite clays in their processes. One successful example of the use of clay in the treatment of coloured effluents is the use of Macrosorb (trade name for a synthetic, hydrotalcite inorganic clay) developed by Crosfield and Unilever Research (Cooper, 1995). The produced sludge is disposed of as slurry to sewers and the treated effluent from this process is suitable for recycling in some dyehouse processes.

Corncobs, rice hulls and other agricultural waste are also potential dye sorbents. Their advantage being that they are plentiful and cheap materials and hence regeneration is unnecessary (Robinson *et al*, 2001).

Membrane filtration has interesting characteristics such as resistance to temperature, harsh chemical environment and microbial attack. It clarifies, concentrates and separates dye continuously from effluent (Mishra *et al.*, 1993; Xu *et al.*, 1999). Disadvantages lie in the disposal of the resulting concentrated residues, the risk of clogging and membrane replacement. It can be successfully used for water recycling in a textile dye plant, especially when the effluent has low dye content.

Ion exchange has not been frequently used in the treatment of dye effluent, as it cannot be applied to disperse dyes (Mishra *et al.*, 1993; Slokar *et al.*, 1998). There is no loss of ion exchange adsorbent during the process, but the

technique is expensive because of the cost of solvents required for regeneration of ion exchange capacity.

2.3.3 Biological treatments

2.3.3.1 Decolourisation by white-rot fungi

White-rot fungi are organisms capable of degrading lignin (Barr *et al.*, 1994). The most studied white-rot fungus is *Phanaerochaete chrysosporium*, which can degrade dioxins, polychlorinated biphenyls (PCBs) and other chloro organics (Chao *et al.*, 1994; Reddy, 1995). It has been reported in the literature that fungi have been used to treat contaminated soil (Davis *et al.*, 1993), and efficiently decolourise textile effluent (Kirby, 1999). White-rot fungi produce enzymes such as lignin peroxidases (LiP) and manganese dependent peroxidases (MnP), which can degrade dyes. Azo dyes (the largest class of commercially available dyes) are not all degraded by micro-organisms, but it has been shown that most of them are degraded by *P. chrysosporium* (Paszczynski *et al.*, 1995). Other types of fungi have been found capable of decolourising dye effluent, e.g. *Hirschioporus larincinus, Inonotus hispidus, Phlebia tremellosa* and *Coriolus versicolor* (Banat *et al.*, 1996; Kirby, 1999).

2.3.3.2 Decolourisation by microbial cultures

Mixed bacteria sampled from different habitats have also been shown capable of decolourising azo dye molecules (Knapp *et al.*, 1995). Decolourisation of dyes has been reported using anaerobic bacteria in 24h-30h incubation using either bacteria in suspension or in biofilms on different support materials (Nigam *et al.*, 1995; Nigam *et al.*, 1996).

Much research has been carried out to investigate azo dye degradation by bacteria. Under aerobic conditions, azo dyes are not degraded, although certain *Pseudomonas* strains might be able to degrade dyes under those conditions

(Kulla, 1981). Intermediates are usually formed during these degradations, but the dyes are not completely mineralised.

Under anaerobic conditions, azo dyes are reduced by numerous bacteria through the activity of nonspecific, soluble enzymes called azo reductases. Azo degradation using azo reductases results in the formation of aromatic amines, which may be toxic, mutagenic and, or carcinogenic (Brown *et al.*, 1993; Sweeney *et al.*, 1994). It is interesting to note that non-specific anaerobic bacteria are capable of degrading a wide range of sulfonated and non-sulfonated azo dyes.

Anaerobic decolourisation of azo dyes and other water-soluble dyes involves oxidation-reduction reaction with hydrogen (instead of oxygen in aerobic conditions). Azo dyes behave as oxidizing agents for the reduced flavin nucleotides of the microbial electron chain and are reduced and decolourised with the re-oxidation of the reduced flavin nucleotides. The azo dye usually acts as the final electron acceptor. The electrons react with the dye molecule by reducing the azo link, hence causing decolourisation (Carliell *et al.*, 1996). The azo reduction of textile dyes under anaerobic conditions does not mineralise them, but has been shown to generate toxic amines (Banat *et al.*, 1996). Hence careful monitoring of the treated effluent should be carried out before release into the environment.

Aerobic decolourisation of a simple azo compound has been reported by Kulla (1981). Studies conducted by Giolando (1992) speculate that aerobic microbial degradation of azo dyes could occur through the biochemical cleavage of the azo bond, leading to the formation of metabolites other than aromatic amines. Aerobic decolourisation of azo dyes have also been reported by Ghoropade *et al.* (1993), Jian *et al.* (1994), Coughlin *et al.* (1997) and Tepper *et al.* (1997), who stated that decolourisation only occurred with low concentrations (4.5 –15 mg/l) of some azo dyes such as acid orange 7, acid orange 8, Acid Yellow 151, Acid Yellow 36, Acid Red 18. Nevertheless, only acid orange 7 and 8 were mineralized. Aerobic conditions are more commonly used to further degrade

products of anaerobic azo reduction, and thus reduce the toxicity (Sheshadri *et al.*, 1994). According to Chung *et al.* (1992), the reduction of azo dyes can be mediated by facultative as well as obligate anaerobes. Many researchers have also demonstrated decolourisation of azo dyes using *Pseudomonas* spp. (aerobic gram-negative chemoheterotrophs) under anaerobic conditions (Chang *et al.*, 2001; Yu *et al.*, 2001; Chen, 2002 and Isik *et al.*, 2003).

The adsorption or accumulation of chemicals onto biomass is called biosorption (Hu, 1992, 1996; Tsezos *et al.*, 1989; Kumar *et al.*, 1998). Dyes have been shown to adsorb onto dead bacteria, yeast and fungi, hence their use in decolourising dye effluents. It has also been noticed that certain dyes have a particular specificity for some microbial species. The advantage of using biomass as dye sorbent is that it can be used to treat toxic dye effluent. This technique is useful and efficient when the conditions are not suitable for growth of the microbial consortium (Modak *et al.*, 1995). Biomass adsorption takes place by ion exchange and is a quick process, e.g. a few minutes in algae to a few hours in bacteria (Hu, 1996).

2.3.3.3 Enzymatic treatments

Enzymatic processes are normally placed between physico-chemical and biological processes, as they involve chemical processes based on the action of biological catalysts.

A lot of research on developing enzymatic treatment systems for solid, liquid and hazardous wastes has been reported in the literature. There are a wide variety of enzymes available from a large selection of plants and microorganisms.

There are three main reasons for using enzymes in waste treatment (Karam *et al.*, 1997): (a) It is essential to develop alternative treatment methods that are cheaper, faster and more reliable than those currently used. (b) Enzymes have specific substrates; hence can be used to target specific pollutants. (c) Recent

biotechnological improvements have permitted mass production of cheaper enzymes through better isolation and purification procedures.

The potential advantages for using enzymatic treatment compared to conventional treatment methods are (Nicell *et al.*, 1993a):

- Treatments can be operated at high and low contaminant concentrations
- Treatments can be operated over a wide range of pH, temperature and salinity
- Absence of shock loading effects
- Absence of delays associated with the acclimatisation of biomass
- Reduction in sludge volume (no biomass generated)
- Process easy and simple to control

Aromatic compounds including phenols and aromatic amines are major pollutants that are found in wastewaters of varied industries such as coal conversion, petroleum refining, textile processing, synthesis of dyes and other chemicals, wood preservation, pulp and paper making (Nicell *et al.*, 1993b). Phenolics are highly toxic pollutants that need to be removed from effluent before discharge. It has been reported that enzymes operate over a broad aromatic concentration range and require low retention times compared to other treatment methods (Siddique *et al.*, 1993). Numerous enzymes have been successfully used as potential alternatives to conventional methods.

The main types of enzymes used to treat phenolic compounds are peroxidases and polyphenol oxidases. Peroxidases are oxidoreductases produced by various microorganisms and plants, and require activation by peroxides such as H_2O_2. Examples of peroxidases include Horseradish peroxidase (HRP), which catalyses the oxidation of various aromatic compounds (e.g. phenols, biphenols, anilines, benzidines), and is active over a broad range of pH and temperature. Lignin Peroxidase (LiP) is another type of peroxidase, and is part of an extracellular enzyme system of the white rot fungus *Phanaerochaete chrysosporium* (Aitken *et al.*, 1989; Venkatadri *et al.*, 1993). It was shown to have a similar mechanism to HRP. LiP's stability was improved at high pH, at

high enzyme concentration and in the presence of its substrate veratryl alcohol and these are the optimum conditions for the removal of phenolics (Aitken *et al.*, 1989).

Other types of peroxidases have also been reported to catalyse the oxidation of mono-aromatic compounds and aromatic dyes: Manganese peroxidase (MnP), chloroperoxidase (CPO), and haemoglobin as an HRP substitute (Chapsal *et al.*, 1986). Polyphenol oxidases are another family of oxidoreductases catalysing oxidation reactions of phenolic compounds. There are two subclasses of polyphenol oxidases: tyrosinases and laccases. Both groups need activation by oxygen.

Tyrosinases catalyse two consecutive reactions of hydroxylation and dehydrogenation of phenols producing quinones, which are unstable and go through non-enzymatic polymerization forming water-insoluble substances, easy to remove by filtration (Atlow *et al.*, 1984; Wada *et al.*, 1993; Sun *et al.*, 1992). It has been reported by Wada *et al.* (1993) that combination of immobilized tyrosinase with chitosan (derived from chitin, a shellfish polysaccharide) for the treatment of phenols yielded a 100 % phenol removal within two hours treatment. Despite this effective means of toxic phenols removal, exploitation is restricted by the high cost of the enzyme.

Some enzymes have been found capable of degrading surfactants, which can cause significant pollution problems and undesirable effects such as foaming in effluents of shampoo and domestic fabric washing formulation factories as well as textile industries using wetting and scouring agents, dyeing auxiliaries and softeners (Thomas *et al.*, 1991). It has been reported that immobilised alkylsulfatase from *Pseudomonas* C12B is able to degrade surfactants (Siddique *et al.*, 1993; Thomas *et al.*, 1991), and shows promise for future use in the treatment of surfactants contained in waste streams.

Table 2.5 shows a list of some enzymes with their potential application for waste treatment. The purpose of using enzymes in waste treatment is to

completely remove specific pollutants or to transform them into by-products. In certain cases degradation products from enzymatic activity are more toxic than parent compounds, hence the necessity of studying toxicity of by-products. This is, however, a laborious process since identification of by-products is difficult. Their disposal might pose a problem, although efforts are being made in industrial recycling (e.g. recovery of metals).

The production of enzymes is expensive because of the cost of their isolation, purification and production. When commercial development leads to large-scale production of an enzyme, its cost is significantly decreased. In most cases, it has been found that immobilized enzymes on solid supports have better activity than free enzymes. Advantages of immobilisation are improved stability, possibility of continuous processing and enzyme re-use (Karam *et al.*, 1997). It is important to consider that the use of enzyme is not advantageous when wastes contain high concentrations of various organic materials and problematic chemicals. Enzymatic treatment might also become an expensive alternative when enzymes are required and produced in large quantities (Aitken, 1993).

Enzymes can catalyse reactions with specific pollutants to remove them by precipitation or transformation to other products. It also can change the characteristics of a given waste to make it more treatable or allow bioconversion of waste material into value-added products. It is important to study reaction by-products, disposal of residues and enzymatic treatment costs before applying enzymes in waste treatment.

Table 2.5 List of some enzymes and their potential applications (from Karam *et al.*, 1997).

Enzyme	Source	Applications	References
Chloro-peroxidase	*Caldariomyces fumago*	Oxidation of phenolic compounds	Aitken *et al.* (1994)
Cyanidase	Isolates from *Alcaligenes denitrificans*	Cyanide decomposition	Basheer *et al.* (1992, 1993)
Cyanide hydratase	Fungal, e.g. *Gloeocercospora sorghi, Stemphylium loti*	Cyanide hydrolysis	Basheer *et al.* (1993)
Haemoglobin	Blood	Removal of phenols and aromatic amines	Chapsal *et al.* (1986)
Laccase	Several fungi , e.g. *Rhizoctonia praticola, Fomus annosus, Trametes versicolor*	Removal of phenols, decolourisation of Kraft bleaching effluents, binding of phenols and aromatic amines with humus	Bollag (1992), Bollag *et al.* (1988), Milstein *et al.* (1988) and Lankinen *et al.* (1991)
Lignin peroxidase	*Phanaerochaete chrysosporium*	Removal of phenols and aromatic compounds, decolourisation of Kraft bleaching effluents	Aitken *et al.* (1994), Cornwell *et al.* (1990), Ferrer *et al.* (1991), Pellinen *et al.* (1988)
Lipase	Various sources	Improved sludge dewatering	Thomas *et al.* (1993)
Lysosyme	Bacterial	Improved sludge dewatering	Hakulinen (1988)
Mn-peroxidase	*P. chrysosporium*	Oxidation of mono-aromatic phenols and aromatic dyes	Aitken *et al.* (1989), Aitken *et al.* (1994)
Peroxidase	Horseradish roots, water-hyacinth, tomato, white radish, soybeans, *Coprinus macrorhizus*	Removal of phenols and aromatic amines, decolourisation of Kraft bleaching effluents, sludge dewatering	Siddique *et al.* (1993), Nicell *et al.* (1993a), Nicell (1994), Klibanov (1982), Klibanov *et al.* (1980), Nakamoto *et al.* (1992)
Phosphatase	Citrobacter sp.	Removal of heavy metals	Macaskie *et al.* (1984, 1987)
Tyrosinase	Mushroom	Removal of phenols	Bollag (1992), Atlow *et al.* (1984), Wada *et al.* (1993), Sun *et al.* (1992)

2.4 Biodegradation and toxicity of dye degradation products

2.4.1 Azo dye reduction

Biological degradation of azo dyes under aerobic and anaerobic conditions has been reported in the literature. In both conditions, reductive fission of the azo group is thought to be the initial step in dye degradation (Meyer, 1981). Chemical reduction of dyes has also been reported in the literature, but was observed to be reversible especially with azo dyes (Gubser, 1972). Reductive fission of azo groups can be carried out by reducing agents such as sodium sulfide, stannous chloride, sodium hydrosulfite, titanous chloride, zinc dust, and Raney nickel. Reduction leads to colour loss (Levine, 1991). Nevertheless, this chemical reductive decolourisation partially reverses on atmospheric exposure.

A wide variety of anaerobic bacteria are capable of degrading azo dyes into aromatic amines, e.g. faecal anaerobes can produce carcinogenic amines such as benzidine and 4-aminoaniline from tartrazine (azo dye) in the human gut (Brown *et al.*, 1993; Chung *et al.*, 1992). Aromatic amines can be mineralised by means of aerobic treatment (Easton, 1995) by non-specific enzymes, through hydroxylation and ring-opening of the aromatic compounds (Zissi *et al.*, 1996). Brown *et al.* (1983) have studied the aerobic degradation of a range of aromatic amines. They found that they were rapidly degraded (> 90 %) and were unlikely to remain in the environment. Therefore anaerobic followed by aerobic treatment can be used to decompose toxic and carcinogenic compounds.

2.4.2 Toxicity

There is a concern that the aromatic amines could pose a more serious toxic hazard in the environment than the intact dye molecules. Over a hundred years ago, Rehn (1895) reported the link between aromatic amines and human cancer, especially bladder cancer among workers from the aniline dye industry.

Carcinogenicity of azo dyes was studied in detail by American scientists in the 1940s. The investigations led to a better understanding of carcinogenicity of many types of azo dyes in rats. Researchers such as Walpole *et al.* (1958) carried out synthesis of methyl analogues of 4-aminobiphenyls (used in the rubber industry), assuming that methylation would decrease carcinogenicity. They discovered that 3-methyl-4aminobiphenyl and related substances were in fact more toxic and gave rise to compounds that induced colon cancer (Weisburger, 2002). Dimethylaminoazobenzene (DAB) and its various derivatives were the first chemical carcinogens to have been thoroughly studied (Levine, 1991).

Azo reduction is usually a key step in the carcinogenic activation of most azo dyes. A better understanding of the mechanism and location of this reductive metabolism is important. Azo reductase activities usually take place in the intestine and in mammalian liver (Brown *et al.*, 1993). Dye reduction is also sensitive to oxygen. It is important to note that a normal liver is mainly aerobic and intestinal microorganisms are in an anaerobic environment. Lipophilic dyes such as the carcinogen DAB are activated by oxidation and conjugating systems. It is interesting to note that the reduction of carcinogenic dyes cancels their carcinogenic activity, whereas the reduction of highly charged water-soluble dyes yields mutagenic products.

Most of the azo dyes used commercially may not have cytotoxic, mutagenic or carcinogenic properties. There is nevertheless no doubt that intestinal azo reductases catalyse the cleavage of the azo link and produce toxic aromatic amines (Fig. 2.2). Soluble azo dyes ingested are believed to be metabolised by the intestinal micro-organisms, whereas the insoluble azo dyes are taken care of by the azo reductases in the liver, which is thought to be a detoxification reaction (Miller *et al.*, 1945; Miller *et al.*, 1948; Miller *et al.*, 1957). Reductive cleavage of the azo bond is probably the most important metabolic reaction of azo compounds. Walker et al. (1971) and Brown (1977) have suggested that azo reduction could occur biologically as a non-enzymatic reaction where one or more flavins may act as electron shuttles.

Far from being fully understood, carcinogenic activation remains to be further studied. It seems that oxidative and reductive pathways produce toxic compounds. Assessments of toxic azo dyes must take into account all pathways, as well as oxygen sensitivity of azo reduction. This is important in the treatment of waste from industrial plants, which mainly use soil bacteria to catalyse reduction aerobically.

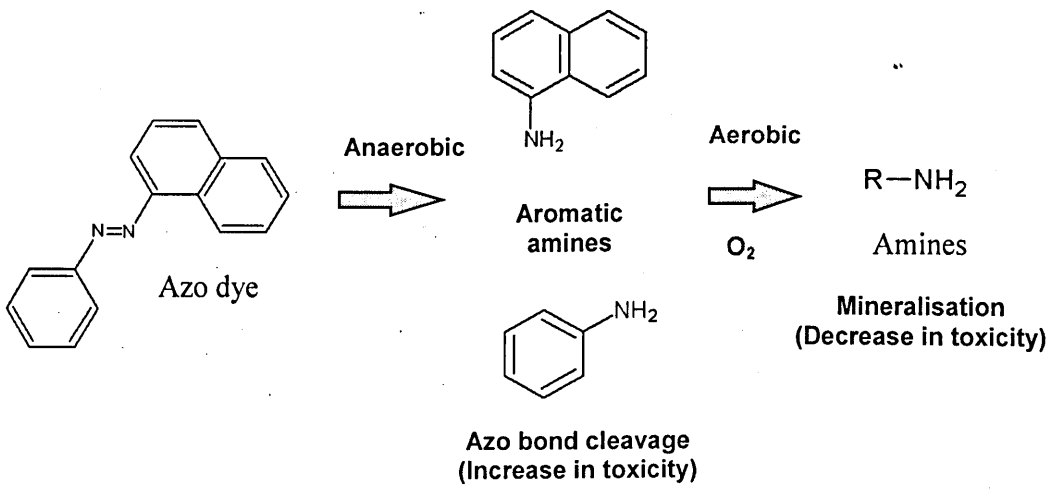


Figure 2.2 Diagram of azo reduction and mineralisation of an azo dye

2.4.3 Azo reductases

Roxon *et al.* (1966; 1967) isolated a pure culture of *Proteus vulgaris* to study the azo reduction of tartrazine. They found that the azo reductase of *P. vulgaris* was a NADPH-specific flavoprotein (Table 2.6).

Table 2.6 Intestinal bacteria reported to produce azo reductase (from Chung *et al.*, 1992).

Organism	references
Acidaminococcus fermentans	Chung *et al.* (1978), Brown (1981)
Enterobacter aerogenes	Soleim *et al.* (1972)
Bacillus sp.	Soleim *et al.* (1972)
Bacteroides sp.	Soleim *et al.* (1972), Azad Khan *et al.* (1983) and *Rafii et al.* (1990)
Butyrivibrio sp.	Rafii *et al.* (1990)
Citrobacter sp.	Chung *et al.* (1978)
Clostridium nexile	Rafii *et al.* (1990)
C. clostridiiforme	Rafii *et al.* (1990)
Enterococcus faecalis	Scheline *et al.* (1970) and Collins *et al.* (1972)
Escherichia coli	Soleim *et al.* (1972) and Azad Khan *et al.* (1983)
Eubacterium sp.	Rafii *et al.* (1990)
E. hadrum	Rafii *et al.* (1990)
Pneumococcus sp.	Azad Khan *et al.* (1983)
Proteus sp.	Roxon *et al.* (1966) and Roxon *et al.* (1967)
Proteus vulgaris	Roxon *et al.* (1966), Roxon *et al.* (1967), Soleim *et al.* (1972), Brown (1981) and Azad Khan *et al.* (1983)
Pseudomonas sp.	Soleim *et al.* (1972)
Pseudomonas aerugina	Brown (1981)
Salmonella typhimurium	Brown (1981)
Staphylococcus aureus	Azad Khan *et al.* (1983)
Streptococcus faecalis	Roxon *et al.* (1966), Roxon *et al.* (1967) and Azad Khan *et al.* (1983)

The research carried out by Chung *et al.* (1978) suggests that an extracellular electron shuttle is required for azo reduction to occur. The use of flavin mononucleotide (FMN) in their experiments caused an increase in azo reduction. Other types of electron carriers such as methyl viologen, benzyl viologen, phenosafranin, neutral red, crystal violet, flavin adenine dinucleotide (FAD), menadion and janus green also increased azo reduction.

Rafii *et al.* (1990) have isolated eight strains of bacteria with high azo reductase activity from the human gastro-intestinal tract, they identified them as: *E. hadrum, Eubacterium sp., C. clostridiiforme, Butyrivibrio sp., Bacteroides sp., C. paraputrificum, C.nexile and Clostridium sp.* The azo reductase activity was found to be oxygen sensitive and associated with riboflavin (FAD and FMN) in most of these bacteria. They thought that the enzymes were released extracellularly and constitutively.

Studies on the effect of diet on azo reductase activity can be found in the literature. Diet influences the composition of the intestinal microflora both in animals and humans (Chung *et al.*, 1977; Chung *et al.*, 1989). Studies on animals revealed that a meat diet showed higher levels of azo reductases in faeces compared to those of a vegetarian diet (Goldin *et al.*, 1976). Similar conclusions have been drawn for human studies. Goldin *et al.* (1980) states that people eating a "Western-type" diet had a higher level of azo reductase in their faecal flora than those of vegetarians. It has also been observed that the addition of *L. acidophilus* supplement in the diet of meat-eating subjects significantly decreases the activity of faecal azo reductases (Goldin *et al.*, 1977).

Burkitt (1976) proposed the theory of dietary fibre benefits, because the lack of fibre might be the cause of certain cancers of the colon and the rectum. The hypothesis stands as the ingested fibre releases soluble sugars (e.g. glucose) when it reaches the colon. These sugars inhibit azo reductase activity; hence decrease the production of aromatic amines in the intestines, and the risk of developing cancer.

There are various factors affecting azo reductase activity. The enzyme system is unfortunately still not fully understood (e.g. the system of enhancement or repression of toxicity). It is nevertheless essential to try better to understand azo reductase activity in order better to control the reaction catalysed, as it produces mutagens and, or carcinogens that can be very harmful to the environment and public health.

2.5 Conclusion from Literature – Objectives and scope of Research

Compared to other industries, the textile processing industry and dyeing processes in particular are the greatest volume effluent dischargers. They face discharge regulations that are more and more difficult to satisfy. The main bodies controlling discharge of effluents are: the EA and the regional water authorities. Moreover, water resources for the textile industry are becoming

increasingly expensive and limited, especially in the south of England where there is a denser population. The problem of colour is also an issue, and is due to the presence of residual dyes in discharged effluent. Although intact dye molecules show low or no toxicity levels, there is pressure on effluent dischargers to reduce the amount of colour for aesthetic reasons.

Azo dyes are widely used chemicals worldwide. Some of them present carcinogenic properties and potential toxicity to the environment; hence a threat to the public health. Their metabolism has been extensively studied and it was found that both oxidative and reductive pathways are involved in their detoxification and or metabolic activation.

Existing sewage treatment methods are being used in the textile industry to bring effluents to conform to the discharge consents and to help reduce charges made by the authorities. Unfortunately, most of them are "end of pipe" techniques, struggling to satisfy the objectives. Some are expensive and others involve production of sludge that contains non-degraded dyes which has to be disposed of by unacceptable methods such as landfill or incineration. The present conventional biological treatments are seen as insufficient, and it is believed that biotechnology can offer complete destruction of dyes with co-reduction of organic compounds, e.g. biological oxygen demand (BOD_5) and chemical oxygen demand (COD). With the present economic situation, European textile industries face harsh competition against Far East countries where labour costs are inexpensive. Many textile factories can not afford a complete re-structure of their sewage treatment plants. The implementation of new processes for biotechnical treatment of textile effluent could, however, ameliorate the situation as well as save water resources, and improve competitiveness. This was therefore the background to the present research, which formed part of a European project on "Biotechnical treatment and recycling of textile processing effluent" (BIOEFFTEX), and involved partnership between research laboratories and textile processors in five European countries and one associated country.

The EU project aimed to develop enzyme and microbial-based biotechnical processes for treatment and recycling of dyehouse effluent.

The main objectives of the PhD research involve:

- Biotechnical degradation of dyestuffs in effluents
- Potential combination of biotechnical and membrane technology
- Development of appropriate laboratory / industrial equipment
- Water reuse for dyeing without any impact on quality of end-products

These objectives include efforts to improve fundamental understanding of the action of microorganisms in treatment plant bioreactors, the degradation mechanism of different types of dye molecules, and also the effect of other effluent components such as surfactants, metal ions and salts on enzymatic and microbial degradation of colour. The use of the newly developed technology also aimed to provide eco-efficient processes with potential for water reuse and recycling, from which other industries such as paper, plastics, automobile, dyestuff producing and pharmaceutical industries might also benefit.

The proposed research was carried out with the close collaboration of a local dyehouse effluent treatment plant. The characterisation of this effluent is described in Chapter 3 and studies on dye and effluent decolourisation using biotechnical resources were then pursued as described in Chapters 4-7.

CHAPTER 3

QUANTUM CLOTHING LTD STEVENSONS' EFFLUENT TREATMENT PLANT

3.1 Introduction to the existing effluent treatment plant at Stevensons

Coats Viyella Clothing Stevensons (now called Quantum Clothing Ltd Stevensons) was established on the banks of the river Amber (Derbyshire) at the beginning of the last century. Its business is batch-wise preparation, dyeing and finishing mainly of cotton and wool knitwear and some woven garments. Water for these processes is taken from different sources: a borehole on the site, the river Amber and from Severn Trent Water. At the time that this work began, the dyeworks had over a hundred machines operating 24 hours a day and five days a week. The effluent usually includes a mixture of waxes, spinning oils, detergents, auxiliary chemicals and dyes from wool and cotton scouring, dyeing and finishing. The effluent treatment plant is formed by several tanks running parallel to the river Amber (Cooper, 1995).

Originally, the effluent treatment was carried out through mixing and contact with aluminoferric (sodium aluminate and ferric sulfate) used to adsorb some of the impurities. The insoluble compounds in the form of flocs were left to settle in a lagoon, and then the treated effluent was discharged into the river Amber. The sludge obtained from the treatment process was pumped out and dewatered on a filter-press. This treatment process became insufficient as levels of pollution quickly increased over the years.

Treatment technologies meeting national legal standards for effluent discharge were studied and carefully considered. Biological treatment and chemical flocculation were the main techniques considered at the time as they were the most cost effective techniques. The effluent plant chosen was a modified

activated sludge treatment, which was set up by Permutit in the late 1960s (Fig. 3.1).

The dyehouse effluent is collected at the treatment plant and roughly filtered using coarse screens to remove large solids from the wastewater. Smaller size solids such as fibre fragments and other suspended solids are filtered out using fine rotary screen. The effluent is then pumped into a balance tank, the role of which is to mix up the effluent and level the temperature to 38-40°C thus conditioning the effluent for biological treatment.

The mixed effluent is then pumped into activated sludge tanks where it is put in contact with environmental micro-organisms, which absorb some of the effluent's waste compounds. The biomass system in the activated sludge is mainly aerobic as air is injected at the base of the tanks through diffusers. The organic materials contained in the effluent are used by the biomass as sources of carbon, nitrogen and phosphorus necessary for its growth. The contact time is three hours at 40°C. The mixed effluent is then pumped into a clarifier where it is left to settle. The clear water at the top of the clarifier tank is directed to other tanks for further treatment. The settled sludge from the clarifier is recovered and re-aerated for biomass to complete the digestion process. It is then returned into the activated sludge, mixed with fresh effluent.

This system was designed to handle 225 m^3 of effluent per hour, but as production increased flows doubled in size and consequently led to decrease of treated wastewater quality (Cooper, 1995). These changes occurred in the 1980s and were followed by the increased use of reactive dyes in cotton dyeing processes. This resulted in effluent containing hydrolysed reactive dyes that are difficult to biodegrade and hence highly coloured effluent was being discharged into the river. Additional treatment tanks were installed on the site in 1986 in order to remove the residual dyes. These were called Dissolved Air Flotation (DAF) tanks in which selected cationic polymers were added to the effluent to interact with anionic dyes and form large coagulated flocs, which float on the surface of the tank. These flocs were mechanically skimmed off leaving the

clean liquid to pass through a heat exchanger, where the treated effluent was cooled before discharge into the river. The sludge produced from the DAF treatment was usually combined with the sludge from the biological treatment tanks for disposal in agricultural landfills (Cooper, 1995).

Additional modifications to the treatment plant have been made in recent years: a second DAF tank has been installed for preliminary separation of suspended solids to improve the treatment process. The increase in cost of sludge disposal to landfill has also led to the installation of a filter press producing compressed solid cakes that are more convenient to dispose of than slurry. In the original Permutit plant, fly ash, obtained from coal burning, was used as a filter aid. When gas and oil replaced coal for heating boilers, it was necessary to set up alternative filtering systems. Fig. 3.1 shows the diagram of Stevenson's effluent treatment plant after 1986. Previously, the effluent was simply discharged into the river after filtration and biological treatments. Figs. 3.2 and 3.3 show photographs of biological and DAF tanks at Stevensons' effluent treatment plant.

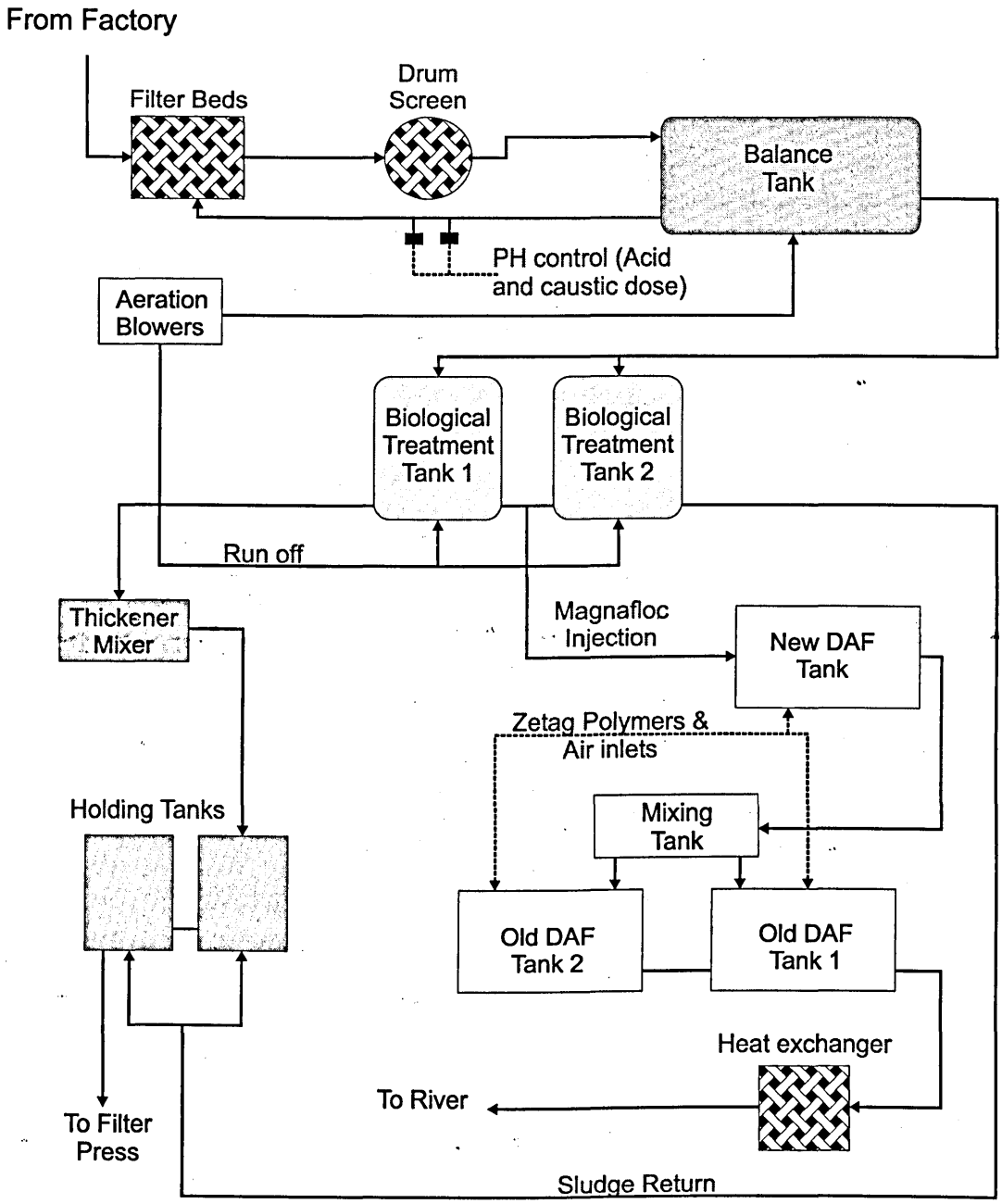


Figure 3.1 Diagram of the effluent treatment plant at Stevensons.

Figure 3.2 Photograph of one of the biological treatment tanks at Stevensons.

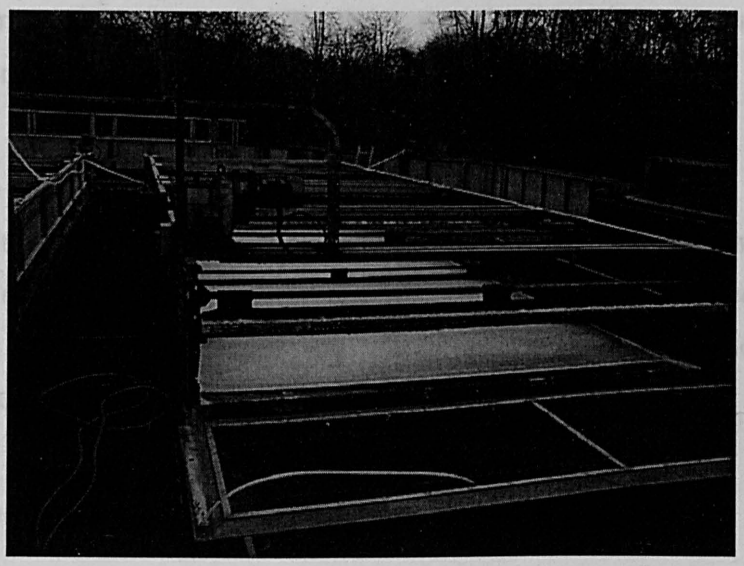


Figure 3.3 Photograph of one of the old DAF tanks at Stevensons.

3.2 Objectives for characterisation of effluent

Textile effluent usually contains a mixture of fibre fragments, auxiliary agents, residual dye compounds from dyeing processes and oil and grease from raw fibres and spinning processes. The chemical composition of effluent varies with the type of dyeworks and processes carried out at the plant. It is most important to characterise the effluent in order to design appropriate treatment methods.

In order to work towards further improvements in Stevensons' effluent treatments, it was necessary to obtain more information about variations in the chemical composition of effluent samples and assess the efficiency of the treatment plant. The characterisation was carried out on the effluent samples collected over different periods of time: over 14 days and within a day to assess the variation in the basic parameters (pH, conductivity, temperature etc). General parameters such as chemical oxygen demand (COD), biological oxygen demand five days (BOD_5), metal ions and non-metal ions concentrations were also determined using available standard methods of analysis.

Further testing has been carried out on samples from different stages of the Stevensons effluent treatment plant collected in June and November 2000. The rest of this chapter describes the analyses carried out, and gives the characteristics of Stevensons' effluent.

3.3 Experimental

3.3.1 Equipment

A Perkin-Elmer inductively coupled plasma atomic emission spectrometer (ICP-AES) model Plasma II was used to analyse and determine the concentration of metal ions in the effluent samples. For each metal ion analysed, standards were prepared (from 0 to 5 ppm). The instrument was calibrated for each metal using

the standard solutions, which emission was measured in triplicates. The metal concentration in the effluent was directly calculated by the instrument.

Principle of the ICP-AES: The plasma is produced by partially ionised argon gas and reaches very high temperatures of up to 10,000°C. Most elements at that temperature emit light of characteristic wavelengths, which can be quantified and used to determine the concentration. The sample for analysis is introduced into the plasma as an aerosol. The light emitted from the different elements is divided into separate wavelengths by a grating and is captured by light-sensitive detectors (Willard *et al.*, 1988).

The HACH DREL/2000 portable spectrophotometer was used to measure nitrate, nitrite, sulfate and COD using the different programs included in the machine and following procedures from the HACH analysis handbook (downloadable from the HACH website, see References).

A Corning flame photometer model 400 was used to measure sodium ions in the effluent samples.

An oxygen electrode with air pump and oxygen controller from Biolab B Braun were used to measure the initial and final dissolved oxygen (DO) of the samples for determination of their BOD_5 values.

A saturated sodium dithionite solution ($Na_2S_2O_4$) and an air-saturated solution of distilled water (using the air pump and oxygen controller) were prepared and used to calibrate the oxygen meter.

A Micro 500 conductivity metre was used to measure conductivity in effluent samples. The electrical conductivity is a measure of the ability of a solution to carry a current and is related to the total concentration of ionized substances dissolved in the water (Willard *et al.*, 1988). It is expressed in micro Siemens per centimetre (µS/cm). A solution of 0.5 M KCl was used to calibrate the instrument.

An ion selective electrode (ISE) from Orion Research Inc. was used to measure chlorides in the effluent samples. The electrochemical sensor develops a potential (E) when placed in an electrolyte solution. The potential developed is related to the activity (a_i) of an ion, hence its concentration.

pH measurements were carried out using a glass electrode pH-metre Hanna Instruments 8520. The pH-meter was calibrated before use with standard buffers at pH7 and 4. The temperature was measured on-site using a thermometer.

3.3.2 Collection of textile effluents from Stevensons

Effluent samples from the balance tank

A mixture of textile effluent was collected from Stevensons. Effluent from the balance tank after the coarse screen was sampled once per day over 14 days. Another 4 samples at 2-hour intervals within one day were also collected to examine the variation of effluent in the balance tank within a day. The basic parameters of these samples, including pH, conductivity, total suspended solids (TSS), dissolved solids (DS), temperature and the flow rate at time of sampling were monitored on site before all the samples were stored in the freezer for further detailed characterisation, as described in Section 3.3.3.

Effluent samples from typical residual dyeing baths

Samples were taken directly from typical cotton dyeing and wool dyeing baths. The samples consisted of the effluents from different steps in the dye cycles, including the exhaust dyebath and other rinsing washing baths. The composition in non-metal ions and residual dye concentrations were determined (Section 3.7).

Effluent samples from different stages of the effluent treatment plant

Samples were taken from the balance tank, from the effluent coming out of the biological treatment tank (or activated sludge) prior to flocculation and from the final discharge to the river. Preliminary analysis was carried out on samples

collected in June 2000. Further investigation was made on samples collected over a period of three days in November 2000 in order to compare with the previous results.

3.3.3 Determination of basic parameters of the effluent

The Flow rate (in litre of effluent processed per second) and the temperature were the only parameters measured on-site the treatment plant.

The total suspended solids (TSS) were determined by measuring the suspended matter from the effluent sample. The sample was filtered through glass fibre filter paper; the residues were dried in a pre-weighed dish at 105°C until constant weight was obtained. The measured increase in weight of the dish corresponds to the total suspended solids. TSS was expressed as mg/L of sample.

The dissolved solids (DS) were gravimetrically measured by filtering the effluent sample through glass fibre and filter paper, then determining the dry weight of the filtrate by difference. The DS was expressed as mg/L sample.

The pH of the samples was measured using a pH-metre. The conductivity of the samples was also measured using the Micro 500 conductivity metre.

3.3.4 Determination of non-metal ion concentrations

All the samples were filtered through a glass fibre and filter paper (Whatman).

Nitrate (NO_3^-), nitrite (NO_2^-) and sulfate (SO_4^{2-}) concentrations of filtrates were determined using the HACH DREL/2000 portable spectrophotometer (25 mL sample cuvettes). The methods of analysis were available as programs in the HACH DREL/2000 spectrophotometer and procedures are described in the HACH analysis handbook.

Nitrate was measured by the cadmium reduction method using the HACH NitraVer5 nitrate reagent powder pillow. NO_3^- is reduced quantitatively to NO_2^- in the presence of cadmium (Cd), which is determined by diazotising with sulfanilamide and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride and form a highly coloured azo dye, proportional to the nitrate present in the sample (HACH analysis handbook).

Nitrite was measured by the ferrous sulfate method using the HACH NitriVer®2 nitrite reagent powder pillow. The method uses ferrous sulfate in an acidic medium to reduce nitrite to nitrous oxide. Ferrous ions combine with the nitrous oxide to form a greenish-brown complex proportional to the nitrite present in the sample (HACH analysis handbook).

Sulfate was also determined spectrophotmetrically by the sulfaVer-4 method using the HACH SulfaVer4 reagent powder pillow. Sulfate ions in the sample react with barium in SulfaVer 4 Sulfate reagent and form insoluble barium sulfate precipitate. The amount of turbidity produced is proportional to the sulfate concentration (HACH analysis handbook).

Chloride ions (Cl^-) content was determined using a chloride-ion selective electrode and NaCl solutions as standards. A buffer solution (0.5 M ammonium acetate and 0.5 M acetic acid in equal quantities) was prepared and mixed with 10 volumes of standards (NaCl) or sample. A calibration curve was obtained with different concentrations of standards (ranging from 0 to 1 g/L NaCl).

Phosphorus (P) content was determined by ICP-AES as described in Section 3.3.5. Standard phosphorus solutions were prepared in Millipore water, ranging from 0 to 5 ppm (the analytical reagent grade standard was obtained from Fisher Scientific).

Ammonia nitrogen was determined by the phenate method, using solutions of ammonium chloride as standards, the procedure was taken from Eaton *et al.* (1995). The principle consists of the formation of an intensely blue compound,

indophenol by the reaction of ammonia, hypochlorite, and phenol catalysed by sodium nitroprusside. The reagents used were phenol solution (11.1 mL liquefied phenol + ethanol to a final volume of 100 mL); sodium nitroprusside 0.5 % (w/v); oxidizing solution: 100 mL alkaline citrate (200 g trisodium citrate and 10 g sodium hydroxide in 1 L) and 25 mL sodium hypochlorite; standard ammonium solutions (ammonium chloride). To a 25 mL sample, 1 mL phenol solution was added, then 1 mL of 0.5 (w/v) sodium nitroprusside solution and 2.5 mL oxidizing solution (with thorough mixing after each addition). Samples were covered with paraffin film; the blue colour was let to develop over one hour at room temperature in subdued light. Absorbance was then measured at 640 nm. Blank and standards (concentration ranging from 0 to 10 mg/L) were prepared and treated the same as samples.

3.3.5 Determination of metal ion concentrations

Samples were passed through a 0.45 μm PTFE filter (Puradisc™, Whatmans). Concentrations of metal ions (Cr, Fe, Mn, Mg, Ca, Zn, Cu) in filtrates were determined by ICP-AES. The instrument standard deviations, based on five replicates, were determined as less than 5 %. This was considered as sufficiently accurate for the examination of single readings. Standard solutions of each ion were prepared at different concentrations in Millipore water, ranging from 0 to 0.1 ppm for Cr, Fe, Mn and Cu, 0 to 1 ppm for Zn, 0 to 100 ppm for Mg and Ca. Analytical reagent grade standards were obtained from Fisher Scientific.

Sodium (Na) concentrations of filtrates were determined, after suitable dilution, by flame photometry; NaCl solutions were used as standards, ranging from 0 to 10 g.

3.3.6 Determination of COD and BOD_5

Chemical oxygen demand (COD) of the effluent samples was determined by the dichromate reflux method with colorimetric determination using the HACH

DREL/2000 spectrophotometer. The COD corresponds to the amount of oxygen in the form of oxidizing agent consumed by the organic water components. The method consists of heating the water sample for 2 hours with a strong oxidizing agent, potassium dichromate. Oxidizable organic compounds react and reduce the dichromate ion ($Cr_2O_7^{2-}$) to green chromic ion (Cr^{3+}).

The reagents needed for a single test were 0.2 g COD catalyst powder, 10 mL de-ionised water, 5 mL 0.250 N potassium dichromate standard solution and 15 mL concentrated sulfuric acid. These reagents were added to 10 mL of effluent sample (diluted if necessary). The resulting solution was put into a reflux apparatus and boiled for two hours on an isomantle. When cooled down to room temperature, COD was measured using the HACH DREL/2000 spectrophotometer set on the COD reflux program. Blank solutions were obtained using distilled water instead of effluent sample and were used to set the spectrophotometer to zero.

Five-day biochemical oxygen demand (BOD_5) was determined after dechlorination using sodium thiosulfite and seeding with Polyseed® (InterBio). This test measures the molecular oxygen consumed during a specified incubation period, for the biochemical degradation of organic material and the oxygen required to oxidize inorganic material, by seeding samples with a sufficient microbial population.

An airtight bottle was filled with sample and incubated at a specified temperature for five days. Dissolved oxygen is measured initially and at the end of the incubation. The BOD_5 is then calculated from the difference between initial and final Dissolved Oxygen (DO).

The reagents needed for the experiment were as follows:
Phosphate buffer solution was prepared by dissolving 17 g KH_2PO_4, 43.5 g K_2HPO_4, 66.8 g $Na_2HPO_4.7H_2O$, and 3.4 g NH_4Cl in 1 L distilled water.

Magnesium sulfate solution ($MgSO_4$) was prepared by dissolving 22.5 g $MgSO_4.7H_2O$ in 1 L distilled water.

Calcium chloride solution ($CaCl_2$) was prepared by dissolving 27.5 g $CaCl_2$ in 1 L distilled water.

Ferric chloride solution ($FeCl_3$) was prepared by dissolving 0.25 g $FeCl_3.6H_2O$ in 1 L distilled water.

These reagents were used to prepare the dilution water (1 mL of each solution in 1 L of distilled water), used to dilute effluent samples. Several dilutions of the effluent samples were carried out in such a way that at least 2 mg/L of oxygen have been consumed after five days incubation. These are the acceptable measuring ranges for BOD_5 according to Rump *et al.* (1988).

The polyseed BOD_5 seed inoculum is a specialised blend of microbial cultures providing a uniform standard for the degradation of industrial wastewater especially designed for BOD_5 measurement. The content of one Polyseed capsule was poured into dilution water for rehydration. It was then aerated and stirred for an hour at room temperature. A volume of 2 mL of the rehydrated seed solution was added to each standard and sample during the BOD_5 procedure.

The oxygen electrode was calibrated before use, with air-saturated distilled water. During DO measurement, temperature and air pressure were also measured (using thermometer and barometer) to convert DO in mg O_2/L. For example, the concentration of oxygen in air-saturated water at 20°C and air pressure of 933 mBars is 8.33 mg/L (Rump *et al.*, 1988). After measurement of initial DO and inoculation, the BOD_5 bottles were closed with the specially designed air-tight lids, then incubated in water bath at 20°C for five days in the dark (to prevent photosynthetic formation of DO).

BOD_5 was calculated according to the formula (Eaton *et al.*, 1995):

$BOD_5 = [(D1 - D2) - (B1 - B2)] / P$

With D1, Initial DO; D2, Final DO (after 5 days incubation at 20°C); B1, DO of seed; control (seeded distilled water) before incubation; B2, DO of seed control after incubation; P, Decimal volumetric fraction of sample used.

3.3.7 Colour measurements

Samples were passed through a glass fibre filter and the filtrates were adjusted to pH 7.0 with sulphuric acid H_2SO_4 or sodium hydroxide NaOH. Absorbance was measured against distilled water over 400 - 700 nm using a Pye-Unicam SP 1800 UV-Visible spectrophotometer.

3.4 Results

3.4.1 General characterisation of the effluent from balance tank over 14 days

The effluent from Stevensons' dyehouse was first passed into the balance tank of the on-site treatment plant. Samples were collected over 14 days and characterised. Table 3.1 shows the basic parameters of the effluent (flow rate, DS, pH, TSS, temperature and conductivity and their variations). During dyeing processes at Stevensons, alkali conditions were required for reactive dyeing of cotton fabrics, and acid conditions for acid dyeing of wool. The mixed effluent obtained from these processes were cooled down in the balance tank and provided average values of pH and temperature of 8.6 and 31°C respectively.

Total suspended solids varied from 105 to 539 mg/L, and dissolved solids from 2617 to 5670 mg/L. Conductivity values were found to be very high, varying from 3489 to 7190 μS/cm. This might be due to the large amount of salt used during dyeing processes. The average flow rate was measured as 66 L/sec. It is noted that some changes had occurred within Stevensons after November

2000, the flow rate was reduced to approximately two-thirds of its original values since the production lines were cut down (given in Table 3.1).

The COD, metal and non-metal ions contents of the effluent samples from the balance tank over 14-days are shown in Tables 3.2 and 3.3. The COD values showed great variations ranging from 110 to 2140 mg/L, which might have been related to the dyeing process carried out; depending on the line of fabric production (e.g. wool scouring). Average concentrations of 131 mg/L chloride, 2.6 mg/L phosphorus, 254 mg/L sulfate contents and high concentrations of sodium ions (7.6 g/L) were found in the effluent. These were due to a large amount of salt and other chemicals needed for buffer systems being used in dyeing processes. Details of other metal ion contents are given in Table 3.3.

Table 3.1 Variation of effluent from balance tank over a 14-day period (basic parameters), with dissolved solids (DS) and total suspended solids (TSS).

Day	Flow (L/sec)	DS (mg/L)	pH	TSS (mg/L)	T (°C)	Conductivity (µs/cm)
1	45	5,392	9.4	170	30	7190
2	73	3,615	8.3	539	30	4820
3	67	4,305	8.8	445	33	5740
4	73	2,617	9.2	210	35	3489
5	68	3,622	9.2	141	30	4829
6	48	5,670	8.8	166	30	7160
7	67	3,400	9.4	129	32	4533
8	76	3,405	8.4	117	33	4540
9	67	2,767	7.5	237	33	3689
10	65	2,872	8.1	429	32	3829
11	58	3,885	9.1	105	30	5180
12	75	4,215	8.5	161	31	5620
13	78	3,232	8.3	132	30	4309
14	64	2,797	7.7	123	30	3729
Mean	**66**	**3700**	**8.6**	**222**	**31**	**4904**
Standard Deviation	**9.9**	**935**	**0.6**	**142**	**1.6**	**1185**

The presence of ammonia, nitrite and nitrate concentrations (2.7, 3.8 and 0.9 mg/L respectively) in Table 3.2 may suggest the occurrence of a nitrogen cycle within the effluent. Ammonia is usually the result of organic or waste mineralisation caused by heterotrophic bacteria. Nitrite is normally produced from the oxidation of ammonia by aerobic bacteria such as *Nitrosomas* spp. through the nitrification process, and nitrate (a less toxic compound) results from the oxidation of nitrite by the *Nitrobacter* spp. (Prescott *et al.*, 1999).

Table 3.2 COD and concentration of non-metal ions (chloride, nitrate, nitrite, phosphorus, ammonia and sulfate) in the effluent samples from the balance tank over 14-days.

Day	COD (mg/L)	Chloride (mg/L)	Nitrate (mg/L)	Nitrite (mg/L)	Phosphorus (mg/L)	Ammonia-N (mg/L)	Sulfate (mg/L)
1	330	88	0	0	2.00	3.78	305
2	330	88	1.2	0	2.29	2.10	220
3	310	221	0	5	3.04	3.26	180
4	290	88	1.8	15	2.77	2.28	355
5	400	88	1.3	0	1.51	3.09	290
6	290	137	1	0	2.10	2.40	200
7	290	133	0	0	1.83	0.81	230
8	850	312	0	5	2.22	3.01	370
9	240	137	3.2	0	4.56	2.92	220
10	220	161	1.1	0	2.09	2.53	215
11	110	88	0	5	2.24	2.23	190
12	1160	88	0.9	0	3.56	2.19	125
13	2140	66	2.4	10	4.07	3.78	355
14	300	137	0	13	1.84	3.26	305
Mean	**519**	**131**	**0.9**	**3.8**	**2.6**	**2.7**	**254**
Standard Deviation	**542**	**66**	**1.0**	**5.3**	**0.9**	**0.8**	**75**

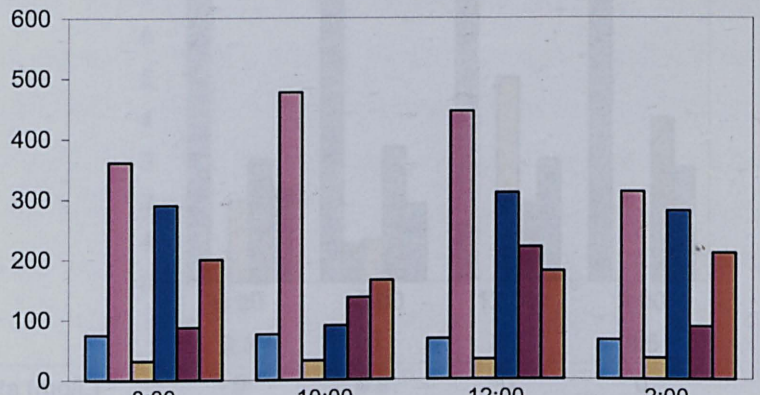
Table 3.3 Analysis of metal ions in the effluent from the balance tank over 14 days.

Concentration of metal ions								
Date	**Cr (mg/L)**	**Fe (mg/L)**	**Mn (mg/L)**	**Cu (mg/L)**	**Zn (mg/L)**	**Mg (mg/L)**	**Ca (mg/L)**	**Na (g/L)**
1	0.03	0.02	0.02	0	0.2	72.9	58.7	6.61
2	0.06	0.03	0.03	0.11	0.3	85.7	100.2	9.53
3	0.07	0.06	0.02	0.12	0.4	73.2	102.3	9.43
4	0.07	0.06	0.02	0.12	0.3	67.6	73.0	8.85
5	0.12	0.05	0.02	0.01	0.4	77.8	64.2	9.24
6	0.04	0.08	0.50	0.19	0.3	132.8	98.0	9.43
7	0.07	0.04	0.01	0.05	0.3	65.3	66.3	8.26
8	0.10	0.08	0.01	0.12	0.3	72.7	96.9	7.58
9	0.11	0.04	0.01	0	0.3	64.2	95.2	5.54
10	0.05	0.04	0.02	0.02	0.3	55.6	73.2	6.51
11	0.08	0.04	0.02	0.10	0.3	76	63.8	8.17
12	0.03	0.06	0.04	0.09	0.3	70.6	89.2	5.25
13	0.08	0.06	0.04	0.13	0.5	34.2	77.0	6.22
14	0.07	0.10	0.02	0.06	1.2	30.7	70.4	5.54
Mean	**0.07**	**0.05**	**0.055**	**0.08**	**0.4**	**70**	**81**	**7.6**
Standard Deviation	**0.03**	**0.02**	**0.13**	**0.06**	**0.2**	**24**	**16**	**1.6**

3.4.2 Variation of the effluent from balance tank within a day

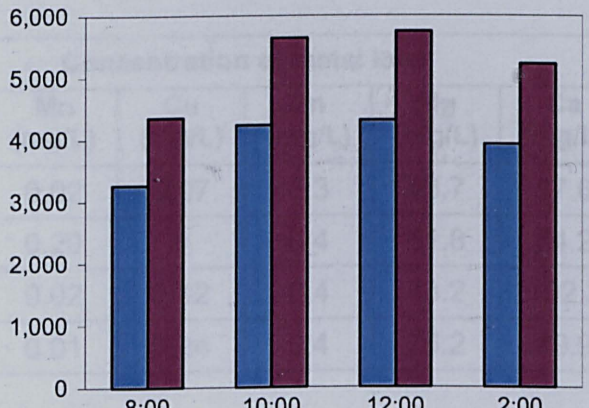
The effluent from the balance tank was also monitored for variation of effluent within a day. Main parameters of the effluent are given in Figs. 3.4 - 3.6 and Table 3.4. Although COD and chloride measurements showed a great deal of variation within a day, the first measurement taken at 8 am was very close to the last one (at 2 pm). Other parameters such as TSS and ammonium also showed significant variations. It is, therefore, difficult to consider one sample collection to be representative of the effluent for the whole day. Nevertheless,

the great variations observed might be related to the weather flooding conditions experienced after November 2000 in Derbyshire.



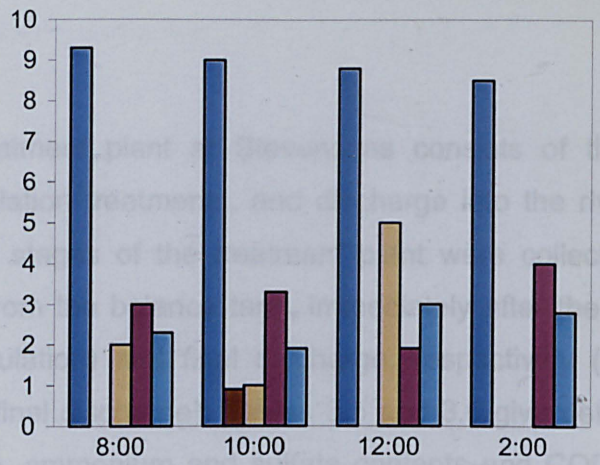
	8:00	10:00	12:00	2:00
Flow (L/sec.)	74.7	76.1	67.2	65.4
TSS (mg/L)	361	477	445	312
Temp. (°C)	32	32	33	35
COD (mg/L)	290	90	310	280
Chloride (mg/L)	87.5	136.7	220.5	87.5
Sulfate (mg/L)	200	165	180	210

Figure 3.4 Variation of effluent from balance tank within a day in flow, TSS, temperature, COD, and concentrations of chloride and sulfate



	8:00	10:00	12:00	2:00
DS (mg/L)	3,262	4,230	4,305	3,915
Conductivity (µS/cm)	4,349	5,640	5,740	5,220

Figure 3.5 Variation of effluent from balance tank within a day in dissolved solids and conductivity.



	8:00	10:00	12:00	2:00
pH	9.3	9	8.8	8.5
Nitrate (mg/L)	0	0.9	0	0
Nitrite (mg/L)	2	1	5	0
Ammonium (mg/L)	3	3.3	1.9	4
Phosphorus (mg/L)	2.3	1.9	3	2.8

Figure 3.6 Variation of effluent from balance tank within a day in pH and concentrations of non-metal ions (nitrate, nitrite, ammonium, phosphorus).

Table 3.4 Variations in concentration of metal ions in effluent from balance tank within a day.

	Concentration of metal ions							
Time	**Cr (mg/L)**	**Fe (mg/L)**	**Mn (mg/L)**	**Cu (mg/L)**	**Zn (mg/L)**	**Mg (mg/L)**	**Ca (mg/L)**	**Na (g/L)**
08:00	0.07	0.05	0.02	0.07	0.3	48.7	67.6	7.29
10:00	0.05	0.03	0.20	0	0.4	61.8	84.2	8.36
12:00	0.07	0.06	0.02	0.12	0.4	73.2	102.3	9.43
2:00	0.06	0.02	0.01	0.24	0.4	75.2	79.9	9.53

3.5 Preliminary analysis of the effluents at different stages of Stevensons' effluent treatment plant

The on-site effluent treatment plant at Stevensons consists of three main stages: biological, flocculation treatments, and discharge into the river. Three samples at the different stages of the treatment plant were collected at the same time. They were from the balance tank, immediately after the biological treatment (prior to flocculation) and final discharge, respectively (duplicates were carried out for the final discharge). Tables 3.5 and 3.6 give details of the metal ions, nitrate, nitrite, ammonium and sulfate contents and COD of these samples. The COD values showed a slight increase (360 and 380 mg/L measured in the final discharge samples). It is not clear it is due to inefficient biological treatment at that time. The cationic polymer added during the flocculation stage may also have contributed to the increase in COD. The concentration in sulfate through Stevensons' effluent was not consistent (random variations between 240 and 360 mg/L). Levels in ammonia, nitrite and nitrate did not show significant variation through the treatment. Variations between the duplicate samples (of final discharge) were not significant except for sulfate (280 and 340 mg/L) (Table 3.5).

Table 3.5 Some parameters of effluents from different stages of the treatment plant. Duplicates were carried out for final discharge.

Date	Sample	COD (mg/L)	Nitrate (mg/L)	Nitrite (mg/L)	Ammonia-N (mg /L)	Sulfate (mg/L)
June 2000	Balance tank	320	< 0	6	4.1	240
	Prior to flocculation	250	5	6	4.0	360
	Final discharge (sample 1)	380	< 0	6	4.5	280
	Final discharge (sample 2)	360	2	4	4.2	340

Table 3.6 indicates that the metal ions Ca, Cu and Mg were not significantly reduced after on-site effluent treatments.

Results obtained from the final discharge samples can be compared to those given by the Environment Agency, who conducted analyses on Stevensons' effluent samples in July 2000 (see Appendix A). The COD value and the concentrations of the metal ions (Cr, Cu and Zn) in the final discharge effluent were found much higher than those from the EA (whose results were 7, 1.97 and 27.3 µg/L for Cr, Cu and Zn respectively and COD was 80 mg/L).

These results were not obtained from the same samples, and only a few tests were carried out. Further testing, on more samples, was therefore carried out to compare the data (Tables 3.7-3.8). Results and discussion are given in Section 3.6.

Table 3.6 Metal analysis of Stevensons' effluents using ICP-AES. Duplicates were carried out for final discharge.

		Concentration of metal ions (mg/L)							
Date	**Sample**	**Zn**	**Mg**	**Ca**	**Cr**	**P**	**Fe**	**Mn**	**Cu**
June 2000	Balance tank	0.6	71.7	64.7	0.2	3.61	0.13	0.06	0.36
	Prior to flocculation	0.3	73.0	75.1	0.19	3.17	0.13	0.05	0.42
	Final discharge (sample 1)	0.1	63.7	82.4	0.05	2.59	0.04	0.03	0.46
	Final discharge (sample 2)	0.2	91.4	104.2	0.06	3.35	0.04	0.04	0.55

3.6 Further testing on effluents at different stages of Stevensons effluent treatment plant

Effluent samples were collected over three days from the three stages of the treatment plant: balance tank, after biological treatment plant (prior to flocculation) and final effluent (before discharge to the river) as described in Section 3.5. Weather conditions were very poor during collection of these samples. Widespread flooding occurred in Britain during November 2000, which affected Derbyshire, where Stevensons is located, resulting in a temporary closure of the treatment plant. As the samples were collected soon after re-starting the plant, it is likely that results from these effluent analyses were affected by the flooding .The main purpose of this testing was to be able to complete, compare and validate the results previously obtained.

Table 3.7 shows concentrations of non-metal ions from the effluent samples. In comparison with the previous analyses carried out in June 2000, lower concentrations of nitrate, nitrite and ammonia, and higher concentrations in sulfate were obtained.

Table 3.7 Some parameters of effluents from different stages of the treatment plant

Sample		Nitrate (mg/L NO_3^-)	Nitrite (mg/L NO_2^-)	Ammonia (mg/L NH_3)	Sulfate (mg/L SO_4^{2-})
14/11/00	Balance Tank	0.8	2	1.8	500
	Prior to flocculation	1.7	3	2.6	425
	Final discharge	0.5	2	9.0	625
15/11/00	Balance Tank	1.1	6	0.4	700
	Prior to flocculation	0.6	5	2.8	400
	Final discharge	0.1	3	11.8	550
16/11/00	Balance Tank	1.7	1	1.5	775
	Prior to flocculation	1.4	4	11.7	400
	Final discharge	0.1	1	22.1	475

A difference can also be noted for ammonia: a significant increase from the balance tank to the final effluent can be observed over the three days. This was not the case in the analysis carried out in June 2000. The high concentration of ammonia measured in the final discharge suggests that the chemical reactions occurring during the flocculation stage may contribute to this increase. A sample of the coagulant used in the flocculation stage (Zetag 7101, a polyelectrolyte coagulant supplied by Ciba®) was analysed but no trace of ammonia was detected. The ammonia increase could, however, be due to the breakdown of the biomass in the activated sludge, hence its release into the effluent. By comparing the metal concentrations (Table 3.8) with those obtained earlier (Table 3.6), most metal ions analysed seem to be in the same range of concentration except for Mg, Cu and Ca, which are lower in recent effluent. The results also show that concentrations of Na and K ions were constant from balance tank to final effluent over the three days. The concentrations of Na determined here are much higher than those previously measured from the balance tank (Tables 3.3 and 3.4), which may have been caused by heavy rain diluting the tanks during the closure of the treatment plant. It could, however, also be due to changes in the production line at Stevensons during that period of time. It was found that the production ratio of wool garment dyeing to cotton was much increased during this period, which would explain the decrease in Na^+ concentration as the dyeing of wool requires less salt.

Table 3.8 Metal analysis of Stevensons' effluents using ICP-AES.

Sample		Concentration of metal ions (mg/L)								
		Zn	**Mg**	**Ca**	**Cr**	**Fe**	**Mn**	**Cu**	**Na**	**K**
14/11/00	Balance tank	0.10	4.6	18.8	0.08	0.02	0.01	0.02	20.4	1.16
	Prior to flocculation	0.09	7.3	29.0	0.04	0.02	0.01	0.03	20.6	1.15
	Final discharge	0.53	8.8	30.7	0.08	0.05	0.03	0.03	20.6	1.15
15/11/00	Balance tank	0.64	7.3	29.0	0.18	0.14	0.03	0.04	20.7	1.15
	Prior to flocculation	0.26	6.9	31.8	0.07	0.16	0.07	0.04	20.6	1.15
	Fina	0.22	6.2	24.3	0.03	0.07	0.05	0.03	20.6	1.02
	l discharge									
16/11/00	Balance tank	0.39	4.2	19.2	0.19	0.11	0.07	0.05	20.5	1.02
	Prior to flocculation	0.19	5.1	29.4	0.04	0.11	0.03	0.05	20.6	1.01
	Final discharge	0.17	5.0	23.9	0.03	0.14	0.05	0.02	20.6	1.01

Table 3.9 shows values for COD, BOD_5 and percentage biodegradability determined in November 2000. It is noted that the COD value for 15/11/00 (prior to flocculation) is unusually high.

On the 15/11/00, Stevensons and its surrounding area suffered from inundation. The heavy rain had affected the biological treatment plant in such a way that the clarifier was no longer doing its job. The biomass transferred from the activated sludge to the clarifier did not settle down and was carried over with the treated effluent to the flocculation stage. The sample collected at this point was indeed heavy in organic matter and could explain the high COD value.

According to the preliminary testing in June 2000 (Table 3.5), there was no reduction in COD in the effluent throughout the treatment process. In contrast, the results from Table 3.9 show lower COD and BOD_5 values in the final effluent, and these reductions were observed over the three-day analysis. The percentage of biodegradability is calculated from the values of the corresponding COD and BOD_5 (BOD_5 / COD ratio). It gives an indication about the effluent's capacity to be further degraded through biological processes. A high percentage of biodegradability indicates that the effluent could be further treated for BOD_5 removal.

Table 3.10 shows that the overall reduction in COD is higher than that in BOD_5. This could be explained by the fact that BOD_5 involves only the oxidation of organic matter by the use of micro-organisms, whereas COD involves a strong oxidation process that includes oxidation of both the organic matter and the inorganic substances.

Table 3.9 COD, BOD_5 and percentage biodegradability

Sample		COD (mg/L)	BOD_5 (mg/L)	Percentage Biodegradability
14/11/00	Balance Tank	475	17.0	3.6
	Prior to flocculation	107	10.2	9.5
	Final discharge	256	2.1	0.8
15/11/00	Balance Tank	542	13.9	2.6
	Prior to flocculation	2680	17.8	0.7
	Final discharge	54	8.1	15.0
16/11/00	Balance Tank	569	15.4	2.7
	Prior to flocculation	288	13.7	4.8
	Final discharge	124	11.4	9.2

Table 3.10 Percentage reduction in COD and BOD_5 based on measurement on balance tank and final effluent samples.

Date	Percentage COD reduction	Percentage BOD_5 reduction
14/11/00	46	88
15/11/00	90	42
16/11/00	78	26
Average	**71**	**52**

3.7 Evaluation of effluent samples from typical residual dyeing baths

3.7.1 Effluent directly from typical dyeing processes including the residual dyebath

The effluents from typical dyeing cycles for dyeing cotton and wool were analysed (Table 3.11). The results show that nitrate and nitrite are only found in wool dyeing baths, which also contain lower level of chloride and sulfate compared to the cotton dyeing baths. The nitrate and nitrite contents might be due to the degradation of micro-protein fragments in the discharge effluent from the wool-dyeing bath.

Table 3.11 Some parameters of effluents from the dyeing baths of cotton and wool

Samples	Nitrate (mg/L)	Nitrite (mg/L)	Chloride (mg/L)	Sulfate (mg/L)	COD (mg/L)	Na (g/L)
Dyeing baths for cotton	0	0	87.5	300	6300	9.63
Dyeing baths for wool	26.3	27	15.1	150	5860	9.53

3.7.2 Typical dyeing processing

The textile wet processing carried out in Stevensons is mainly preparation, dyeing and finishing of cotton and wool fabrics or garments. Direct, reactive and acid dyes are commonly used. Table 3.12 lists the 20 dyes that are currently the most frequently used in Stevensons. Substantial amounts of other chemicals, such as common salt, soda ash, acetic acid, sodium sulphite and surfactants are also used.

Table 3.12 Most common dyes used at Stevensons.

TOP 20 DYES		
	Dye Name	**Colour Index (C.I.)**
1	Neutrilan Black MR	Acid Black 194
2	Everzol B Blue R	Reactive Blue 19
3	Procion Navy HEXL	-
4	Everzol Black RGR	-
5	Neutrilan Navy MB	Acid Blue 193
6	Procion B Red HEGXL	-
7	Evercion Yellow E5R	Reactive Yellow 105
8	Intracron Black VCKN	Based on Reactive Black 5
9	Conactive Black WR	-
10	Evercion Red E4B	Reactive Red 120
11	Lanasol Red B	Reactive Red 65
12	Procion Yellow HEXL	-
13	Cetacid Navy M5R	Acid Blue 113
14	Lanacron Navy SG	-
15	Everzol Yellow 3RS	Reactive Yellow 176
16	Nylanthrene Yellow C3RL	Acid Orange 67
17	Everzol Red PE	-
18	Evercion Navy E2R	Reactive Blue 171
19	Procion Crimson HEXL	-
20	Nylosan Blue NBLN	-

3.8 Colour in effluents from dyeing bath and balance tank

Absorbance of effluent samples was measured against distilled water over 400-700 nm of wavelength using a Pye-Unicam SP 1800 UV-Visible spectrophotometer. Curves 1 and 2 in Fig. 3.7 show the absorbance of samples from cotton and wool dyebaths respectively. Substantial amounts of residual

dyes were found in these samples. For the balance tank, where dyebath effluent is mixed with the effluent from other wet processes, it was found that although the effluent contained a mixture of dyes, absorbance over the range 400-600 nm had decreased.

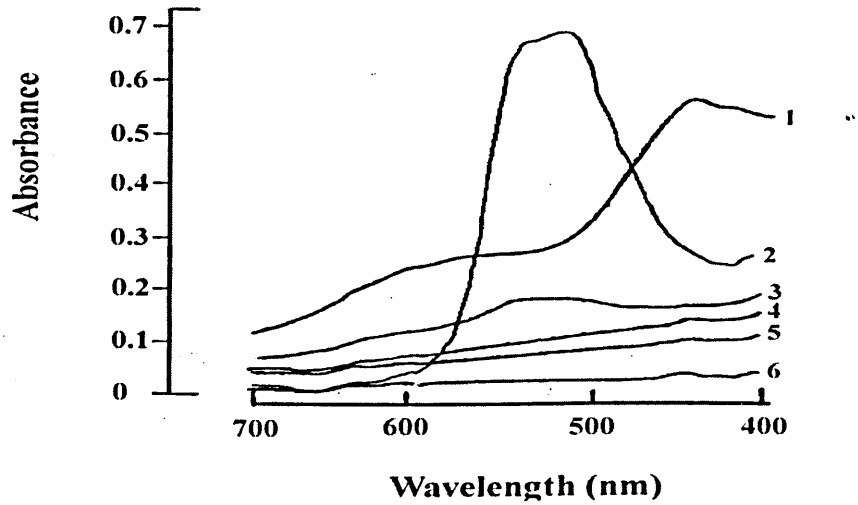


Figure 3.7 Wavelength scan of samples from (1) wool dyeing bath, (2) cotton dyeing bath, (3) balance tank on day 14, (4) balance tank on day 4, (5) balance tank on day 3 and (6) balance tank on day 9.

3.9 Conclusion

Effluent was collected from Stevensons over a 14-day period. The variation in basic parameters of the effluents was analysed and discussed. Further chemical composition of the effluents from the mixed effluent, individual dyebaths and different stages of the effluent treatment plant were determined. This included ICP-AES analysis to determine levels of selected metal ions in the effluents. A database for the effluent from Stevensons was established.

In November 2000, Stevensons dramatically reduced production of garment dyeing and finishing to about two thirds of the previous level and the flow of

effluent was also reduced to about 22 L/second. The recent analysis of Stevensons' effluent has updated information on the basic parameters and ionic composition of the effluent. It was found that the concentrations of most non-metal components in the effluent from the balance tank still remain at the same level except for sulfate, which has increased to 500 - 775 mg/L (Table 3.7). The concentrations of Mg, Ca and Na ions were significantly reduced to different extents: Mg was reduced from 70 to 4.2 mg/L, Ca from 81 to 19.2 mg/L and Ca from 7.6 g/L to 20.5 mg/L (Tables 3.3 and 3.8).

The effluent from the different stages of Stevensons' treatment plant: balance tank, after biological treatment (prior to flocculation) and final discharge were analysed. The information obtained from the effluent characterisation is very useful for the future design of biological treatment processes. During these analyses, it was found that the concentrations of most metal ions did not show great variations through Stevensons' effluent treatment (Table 3.8). COD and BOD_5 decreased through the treatment process (Tables 3.9 and 3.10), but ammonia increased in concentration, from 1.5 to 22.1 mg/L on 16/11/00 (Table 3.7). Results from the analyses should be carefully considered as they may have been affected by the weather (heavy rain and flooding), which played an important role in the functioning of the effluent treatment plant. Table 3.13 shows the average concentrations of BOD_5, ammonia and metals in effluent samples taken over three days (14 to 16/11/00) before and after treatment at Stevenson's treatment plant. The data shows that BOD_5 and metals levels in final discharge comply with the consent limit. The ammonia concentration, on the other hand is too high.

Table 3.13 Average concentrations of BOD_5, ammonia and metals in balance tank and final discharge effluent compared with consent limit. The data was taken from results shown in Section 3.6.

Parameter	Balance tank	Final discharge	Consent limit (from Cooper, 1995)
BOD_5 (mg/L)	15.4	7.2	15
Ammonia N (mg/L)	1.2	14.3	5
Metals (µg/L)	50	55	1000

Following the characterisation of Stevensons' effluent, dye degrading bacteria and enzymes were studied and treatment systems were set up to assess the efficiency of the system (Chapters 4 - 7).

CHAPTER 4

MICROBIAL STUDY OF STEVENSONS' ACTIVATED SLUDGE

4.1 Introduction

4.1.1 Objectives

The isolation from the environment of microorganisms capable of degrading or mineralising pollutants is well documented in the literature (Marchant *et al.*, 1994; Nigam *et al.*, 1996; Forney *et al.*, 2001). According to Wagner *et al.* (2002), microbial communities present in activated sludge or biofilm reactors are responsible for most of the carbon and nutrient removal from sewage and represent the core component in biological waste water treatment plants.

This chapter describes the isolation and identification of dye-decolourising microorganisms taken from Stevensons' activated sludge.

4.1.2 Overview of experimental approach

Stevensons' activated sludge is likely to comprise a large microbial population surviving in symbiosis in the presence of heavily polluted textile effluent. Some of the bacteria, in order to survive, have developed the ability to transform pollutants and use them for growth. As discussed in the literature review, colour is of great concern in textile effluent as it is the most obvious indicator of water pollution. It is essential to develop efficient treatments that remove colour from the effluent before its discharge into the receiving waters. First, it was decided to identify the number of microbial types present in the activated sludge by observing and comparing colonies formed from diluted samples spread on nutrient agar. The total population density of bacteria in the activated sludge was also determined.

Screening tests were carried out to isolate dye-degrading bacteria from the activated sludge samples. The test criteria chosen were the decolourisation of selected anthraquinone and azo dye solutions. Each type of bacterial strain was isolated and sub-cultured to obtain pure cultures of bacteria. Each microbial type was grown in a liquid nutrient medium containing one of the dyes at 50 mg/L, under both aerobic and anaerobic conditions. After a few days incubation at 30°C, the colour of the samples was measured using a spectrophotometer and compared to their initial colour absorbance. Results were expressed in percentage absorbance.

The bacterial strains that caused the lowest percentage absorbance (the most decolourisation) were selected and identified using the analytical profile index (API) 20NE test, a manual biochemical system for the rapid identification of members of the non-*Enterobacteriaceae*.

4.2 Experimental

4.2.1 Equipment

A sample from Stevensons'balance tank was taken to isolate dye-degrading bacteria. It was homogenised using a sonicator (Soniprep 150, MSE), which is a bench mounted ultrasonic disintegrator used for the disruption of fragile cells and separation of aggregated cells. The piezoelectric transducer produces vibrations from an electrical input (usually 23 kHz generation).

Dye samples were incubated in an orbital incubator S150 Stuart Scientific.

A Pye-Unicam SP 1800 UV-Visible spectrophotometer was used to measure the colour absorbances.

The identification of isolated bacteria was achieved using the Analytical Profile Index (API) manual biochemical system, supplied by BioMérieux UK Ltd.

4.2.2 Media and solutions

Media used for decolourisation screening

Nutrient broth composition (supplied by Oxoid): "Lab Lemco" powder (1 g/L), yeast extract (2 g/L), peptone (5 g/L) and sodium chloride (5 g/L).

Nutrient agar composition (supplied by Oxoid): "lab lemco" powder (1 g/L), yeast extract (2 g/L), peptone (5 g/L), sodium chloride (5 g/L) and agar (15 g/L).

Pre-culture of test organisms

Microorganisms were maintained on agar plates (nutrient agar, 30°C, aerobic condition). Liquid culture of bacteria was performed in nutrient broth in culture flasks (at 30°C for 24 hours) and was used as inoculum.

Preparation of dye stock solution:

A concentration of 0.5 % (w/v) dye was prepared in distilled water and kept at room temperature.

Selective *Pseudomonas* agar

Selective *Pseudomonas* agar was prepared using Oxoid *Pseudomonas* agar base (CM55a) from Oxoid Ltd supplemented with one vial (2 mL) of cetrimide-nalidixic (C-N) for selective isolation of *Pseudomonas aeruginosa*. The same Oxoid *Pseudomonas* agar base supplemented with one vial (2 mL) of cetrimide-fucidin-cephaloridine (C-F-C) was prepared for selective isolation of *Pseudomonas* spp.

The *Pseudomonas* Agar Base (CM55a) was prepared by weighing 48.4 g of the Oxoid *Pseudomonas* Agar Base powder and put in 1 litre of distilled water; 10

mL of glycerol were added, then autoclaved at 121°C for 15 minutes. The medium was allowed to cool down to 47°C, then the contents of two vials of the C-N or C-F-C supplement were added and well mixed for immediate pouring into Petri dishes. These procedures were carried out under sterile conditions (media were autoclaved and agar plates were poured near a Bunsen flame).

4.2.3 Isolation of bacteria and determination of their concentration in the activated sludge

A sample of the activated sludge from Stevensons (5 mL) a universal bottle and was shaken vigorously and submitted to sonication for one minute in order to break the polysaccharide matrix, which is the structural architecture of biofilm formed by the bacteria as an extra cellular medium facilitating their adherence.

The bacterial cell density in the activated sludge was determined by serial dilutions of that suspension. Serial dilutions were carried out to 10^{-9} (Table 4.1). Volumes of 0.1 mL were spread from dilution 10^{-4} to 10^{-9} onto triplicate nutrient agar plates. The plates were incubated aerobically at 30°C for two days. The colonies developed on the nutrient agar plates were manually counted and the cell density calculated (Table 4.1). The colonies were also compared to each other on morphological characteristics (colour, cultural appearance) (Table 4.2).

The Gram stain procedure is a method dividing bacteria into two categories Gram-positive and Gram-negative. The difference between them is mainly due to the structure of the microbial cell walls. In Gram-positive bacteria, the cell wall constitutes only a thick peptidoglycan layer on top of the plasma membrane, whereas in Gram-negative bacteria, the cell wall is more complex and is surrounded by an outer lipid membrane. A suspension of a pure culture of bacteria was prepared for each of the isolated strains of bacteria. A smear was made and stained with the dye crystal violet; followed by treatment with an iodine solution, which facilitates interaction between the cell wall and the dye. The smear was then washed using ethanol. At this stage, Gram-positive bacteria retain the crystal violet and stay purple whereas the Gram-negative

bacteria lose the dye and become colourless. Finally, the smear was stained with safranin as a counterstain, which colours the Gram-negative bacteria in reddish-pink.

4.2.4 Assessment of the isolated bacteria for dye colour removal

Three dyes were chosen for the assessment (their structures are displayed in Fig. 4.2):

- C.I. Acid Red 73 supplied by DyStar®
- Intracron black VCKN (C.I. Reactive Black 5) supplied by Yorkshire
- Everzol blue R special (C.I. Reactive Blue 19) supplied by Everlight

The azo dyes C.I. Acid Red 73 and C.I. Reactive Black 5 were selected because they have been reported in the literature to be decolourised through biological treatments (Nigam *et al.*, 1996; Fitzgerald *et al.*, 1995; Heinfling *et al.*, 1998). The anthraquinone dye C.I. Reactive Blue 19 was selected for comparison with the azo dyes. The wavelength of optimum absorbance (λmax) for each dye in solution was determined by running a spectrophotometric scan in the range of 400 - 700nm. Absorbance of dye samples prepared in nutrient broth was measured against nutrient broth as a reference over 400 - 700 nm using a dual-beam scanning spectrophotometer (Fig. 4.1).

The pH was measured for each of the dye solutions: 7.1, 7.2 and 7.2 for C.I. Acid Red 73, C.I. Reactive Blue 19 and C.I. Reactive Black 5 respectively.

The isolated bacteria were individually tested for colour removal by inoculating the dye solutions and incubating under aerobic and anaerobic conditions for a few days. Aerobic conditions were established by using 250 mL capacity Erlenmeyer conical flasks with foam caps incubated at 30°C at 100 rpm in an orbital shaker for seven days. Glass vials (20 mL) sealed with rubber caps were used to obtain anaerobic conditions. Samples were incubated at 30°C without shaking.

The three dyes were prepared in nutrient broth after filtration through a TF 0.45 μm filter to a final concentration of 50 mg/L. The solutions (total volume of 50 mL) were then inoculated individually with the eight isolated microorganisms.

Culture, dye, broth samples were withdrawn over a period of seven days (taken at day 0, 1, 2, 5 and 7) and centrifuged at 13,000 rpm for ten minutes to remove bacteria. The supernatants were analysed for the visible spectra of the dyes using the spectrophotometer set at the λmax of the dyes, which are 520 nm for C.I. Acid Red 73, and 600 nm for both C.I. Reactive Black 5 and C.I. Reactive Blue 19 (Fig. 4.1). These screening tests allowed each isolated bacterium to grow in a nutrient broth medium in presence of a single dye during a week. The initial dye solution prepared at 50 mg/L was considered as a 100 % of the colour. The percentage absorbance shown on the graphs (Figs. 4.3, 4.4 and 4.5) corresponds to the percentage colour remaining in the dye solutions after incubation. The results are expressed as percentage absorbance and were calculated from the absorbance values obtained at the time zero of incubation (A_0) and at the sampling time (A_t), according to the formula: Percentage absorbance = $(A_t \times 100)/ A_0$

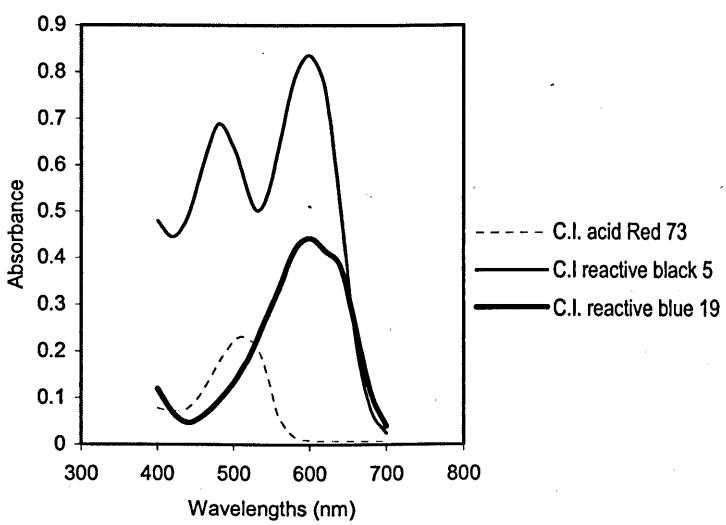
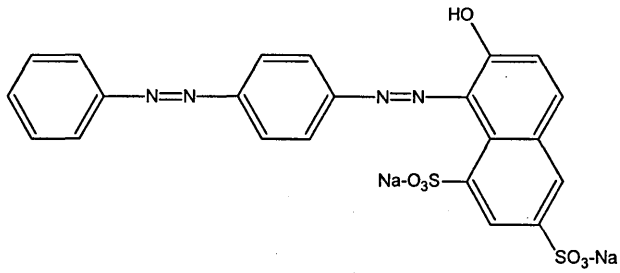
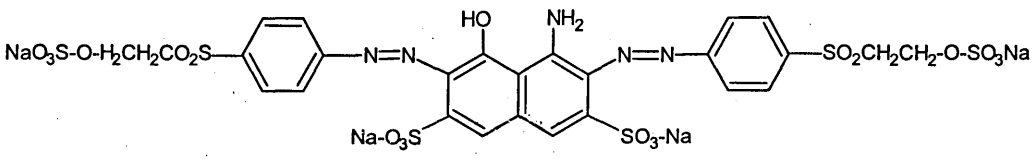


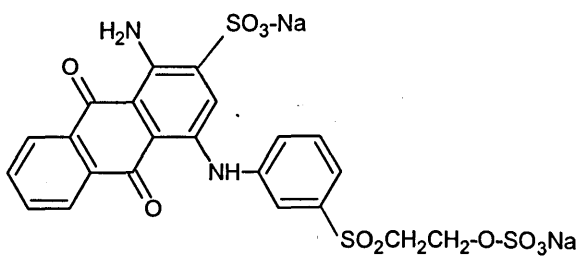
Figure 4.1 Wavelength scans of the dyes C.I. Acid Red 73, C.I. Reactive Black 5 and C.I. Reactive Blue 19



C.I. Acid Red 73



C.I. Reactive Black 5



C.I. Reactive Blue 19

Figure 4.2 Structure of the diazo dyes C.I. Acid Red 73, C.I. Reactive Black 5 and the anthraquinone dye C.I. Reactive Blue 19.

4.2.5 Identification of the isolated dye-decolourising bacteria

One of the most popular methods of bacterial identification is the API 20NE system (Prescott *et al.*, 1999). It is a manual biochemical system for rapid identification of members of the family *Enterobacteriaceae* and other Gram-negative bacteria. Usually, the API 20NE system is more appropriate and specific to the identification of environmental bacteria, e.g. bacteria found in soil or sludge samples. The API 20NE test was supplied by BioMérieux UK Ltd.

The test consists of a plastic strip with 20 micro-tubes containing dehydrated substrates, corresponding to certain biochemical characteristics. The test substrates in the 20 micro-tubes are inoculated with a pure culture of bacteria evenly suspended in sterile physiological saline. After 5 hours or overnight incubation, the 20 test results are converted to a seven or nine digit number profile based on results being positive or negative. The profile number is used with a computer or a book called the *API Profile Index* to find the name of the bacterium.

4.3 Results

4.3.1 Isolation and description of bacteria from Stevensons' activated sludge

Table 4.1 gives the approximate cell concentration of a sample of Stevensons' activated sludge (1.1×10^8 cells/mL calculated from the diluted concentration 10^{-4}). Eight different types of colonies were visually identified based on their morphology. Each of them was sub-cultured separately on nutrient agar to purity. Further observations of the colonies were done by gram stain. Each colony's cell morphology was described using a microscope.

Tables 4.2 and 4.3 describe the morphology of the colonies and cell structure. It is interesting to note that all of the isolated bacteria were Gram-negative.

Table 4.1 Number of colonies on plates and concentration of microbes (in cells/mL).

Concentration	Number of colonies counted	Average concentration (cells/mL)
10^{-9}	0 0 0	-- -- --
10^{-8}	1 1 0	10^{9}
10^{-6}	31 23 26	2.7×10^{8}
10^{-4}	920 1156 1064	1.1×10^{8}

Table 4.2 Description of the cell colonies.

Colony	Surface view	Lateral view	Diameter (mm)	Colour/appearance
1	Crenated	Umbonate	0.6	Opaque with small dark centre
2	Crenated	Umbonate	0.6	Translucent with opaque centre
3	Entire	Umbonate-domed	0.4-0.5	Opaque with small dark centre
4	Entire	Domed	0.3	Translucent
5	Crenated	Raised	0.2	Translucent
6	Entire	Umbonate-domed	0.3	Red opaque
7	Entire	Umbonate	0.8	Translucent with opaque centre
8	Crenated	Umbonate	0.3	Translucent with opaque centre

Table 4.3 Gram stains (the diameters were measured ten days after plate spreading).

Colony	Gram	Cell structure
1	-	Cocci or short rods + bacilli
2	-	Short rods
3	-	Cocci or short rods
4	-	n/a
5	-	Long bacilli
6	-	Rods (some in pairs)
7	-	Rods (often in pairs)
8	-	Small bacilli or rods

4.3.2 Quantitative assessment of decolourisation under aerobic conditions

Figs. 4.3, 4.4 and 4.5 show results for colour removal of three dye solutions (two diazo dyes and an anthraquinone dye) inoculated with eight different bacteria and incubated under aerobic conditions. As most current effluent treatment plants operate aerobically, it would be very interesting to isolate bacterial strains capable of removing all or most of the colour in a dye solution.

Results show that only bacterium eight exhibits low % absorbance after 7 days incubation (40% of colour remaining, see Fig. 4.3), whereas for the other bacteria, the lowest percentage absorbances reach approximately 70 %.

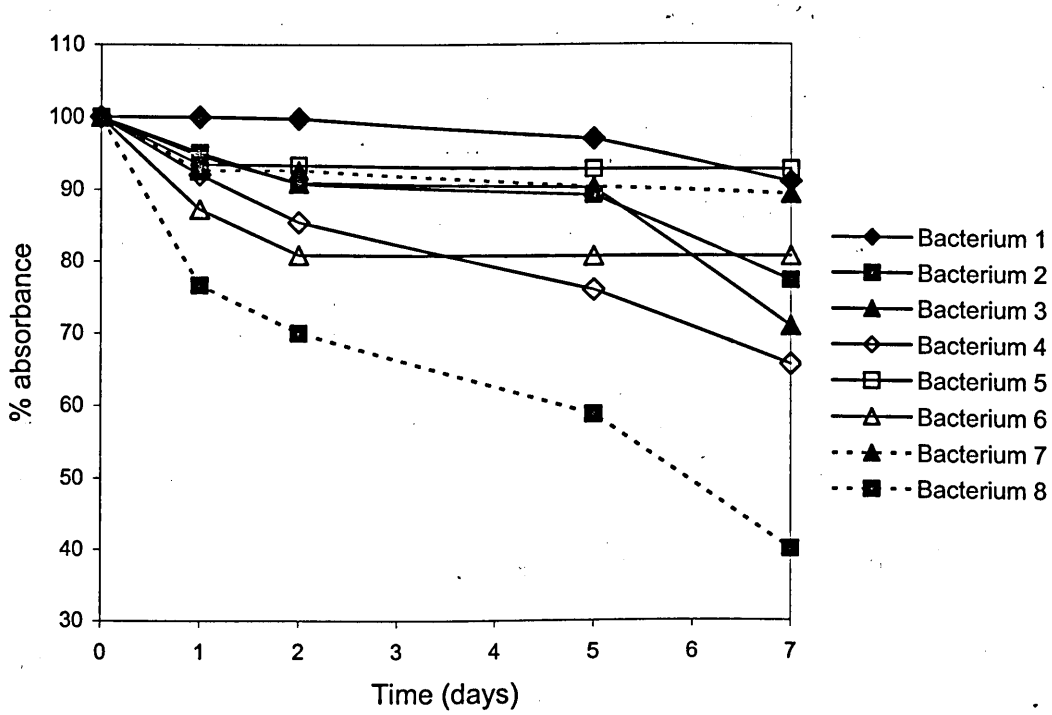


Figure 4.3 Colour removal of C.I. Reactive Blue 19 incubated under aerobic conditions with eight different bacteria isolated from the activated sludge.

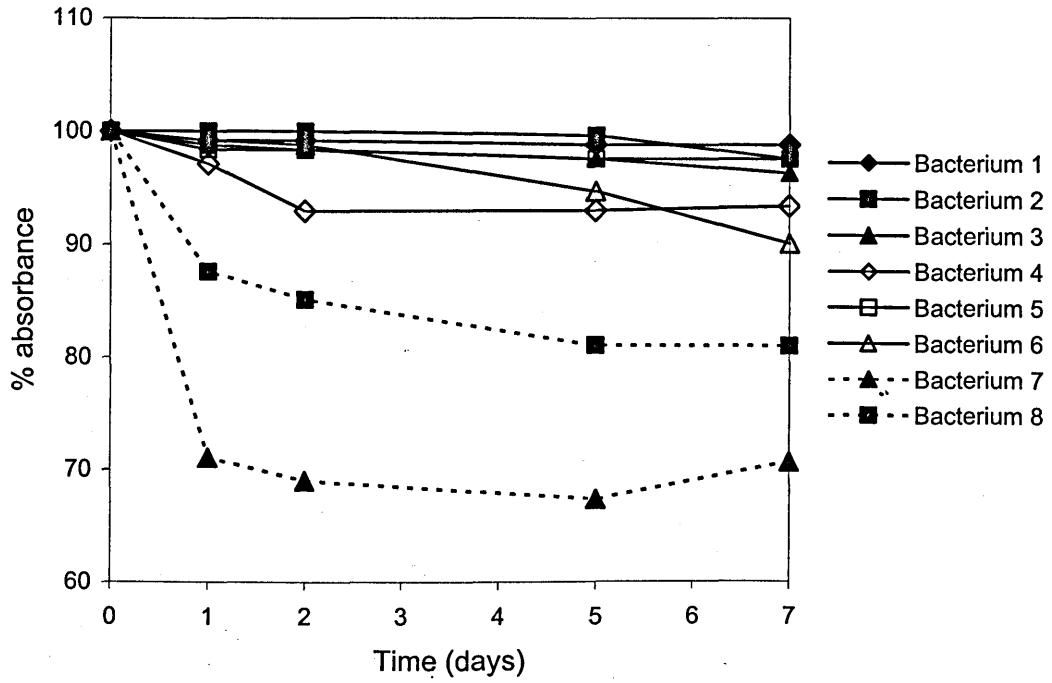


Figure 4.4 Colour removal of C.I. Acid Red 73 incubated under aerobic conditions with eight different bacteria isolated from the activated sludge.

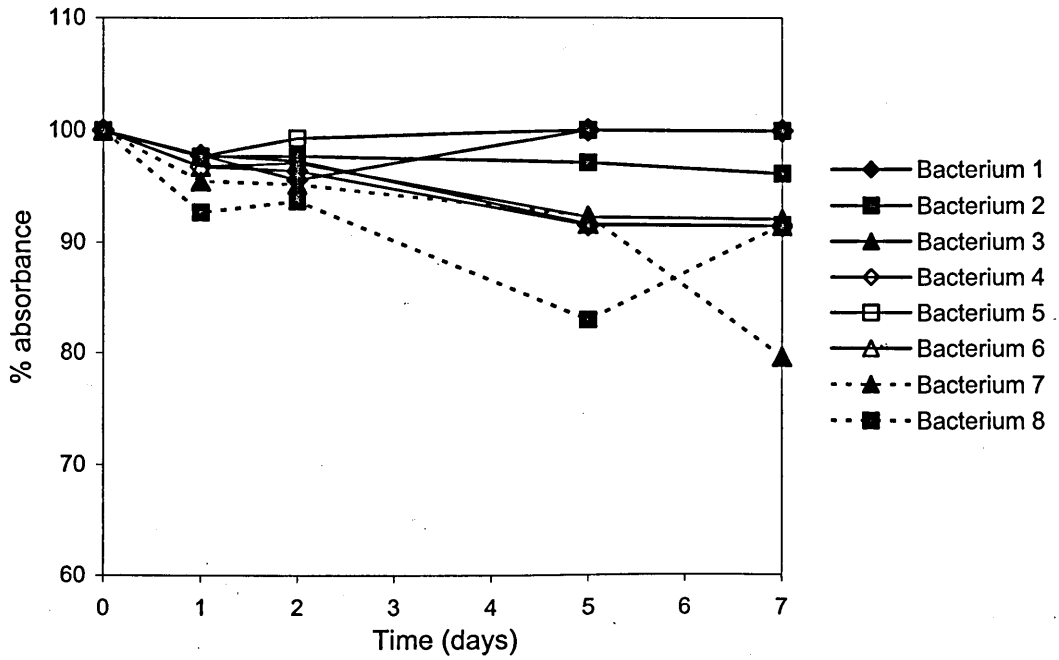


Figure 4.5 Colour removal of C.I. Reactive Black 5 incubated under aerobic conditions with eight different bacteria isolated from the activated sludge.

Some observations can also be made about the colour change in the incubated aerobic flasks. From day 5, the sample containing C.I. Reactive Blue 19 and inoculated by bacterium 8 showed the presence of dark blue lumps of micro-organisms appearing at the bottom of the flask. The same samples inoculated by bacterium 4 and 7 appeared green from day 2 followed by most of the other samples at day 7. The sample containing bacterium 8 was decolourised the most (Fig. 4.3).

Further observations of the biomass colour can be made as the colour removal from the dye solution might have occurred by adsorption of the dye molecule onto the bacteria's membrane, and hence would not be due to enzymatic or metabolic activity from the bacteria. A sample from each flask was taken in Eppendorf tubes and centrifuged at 13,000 rpm for 10 minutes for observation of the colour of the centrifuged pellets of solids. Table 4.4 shows the description of pellets. It is interesting to note that some bacteria adsorb some dyes but not others. Bacterium 2 for example adsorbs C.I. Acid Red 73 but not Reactive Blue

19 nor Reactive Black 5. From these observations, C.I Reactive Blue 19 and Acid Red 73 are adsorbed on to most of the bacteria, especially bacteria 3, 4, 5, 6 and 8. It was considered to be not essential to quantitatively determine the amount of colour that had been adsorbed as the decolourisation experiments showed low colour removal.

Table 4.4 Description of pellets taken after centrifugation of the aliquots

Bacterium	C.I. Reactive Blue 19	C.I. Acid Red 73	C.I. Reactive Black 5
1	Bright yellow with blue layer	White with thin red layer above	White
2	Whitish yellow with thin layer of dark green	Red	Pale brown
3	Dark blue	Yellow with thin red layer above	Pale brown
4	Yellow with thick layer of blue	Red	Pale brown
5	Dark blue	Pink with thin red layer above	Pale brown
6	Dark blue green	Pale pink	Red
7	White with thin layer of green	Pink with thin red layer above	Brown
8	Dark blue	Red	Grey

4.3.3 Quantitative assessment of decolourisation under anaerobic conditions

Fig. 4.6 shows the colour removal of C.I. Reactive Blue 19 solutions by eight different bacteria under anaerobic culture conditions. From day 2 of incubation, some of the vials showed an obvious colour change. These were vials inoculated with bacteria 2, 4 and 8 for the C.I. Acid Red 73 solution, which turned from red to orange (Fig. 4.7).

Compared to both azo dyes, C.I. Reactive Blue 19 did not show much decolourisation. The lowest percentage absorbance found was 65 % with bacterium 2 (Fig. 4.6). On the other hand, the diazo dyes show good results

with only 3 % absorbance (after 7 days incubation) being measured with bacterium 4 in C.I. Acid Red 73 solutions.

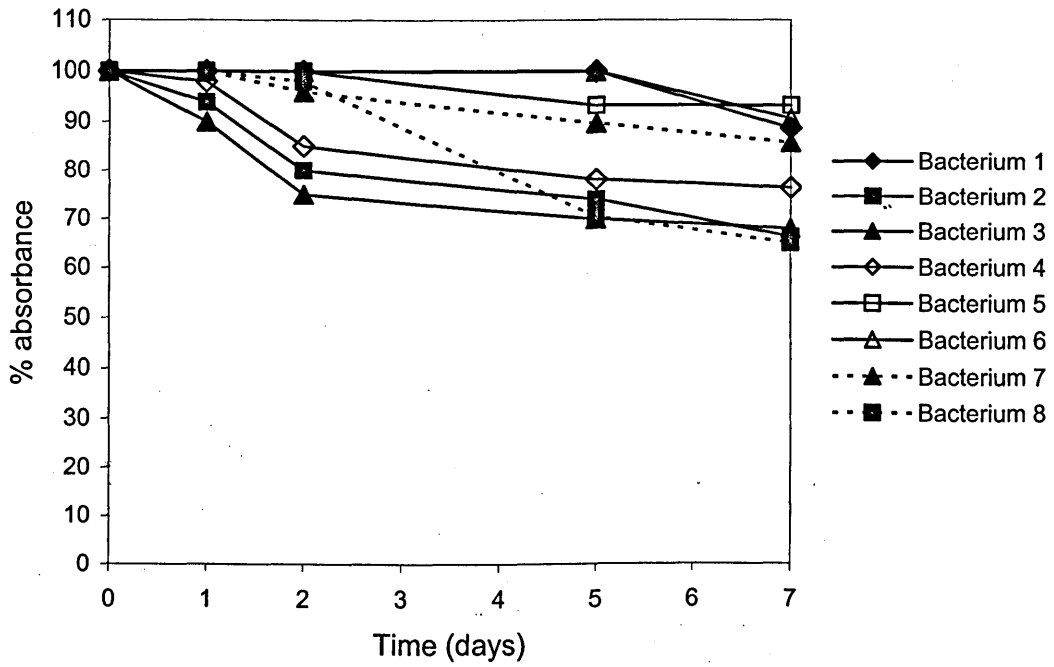


Figure 4.6 Colour removal of C.I. Reactive Blue 19 incubated under anaerobic conditions with eight different bacteria isolated from the activated sludge.

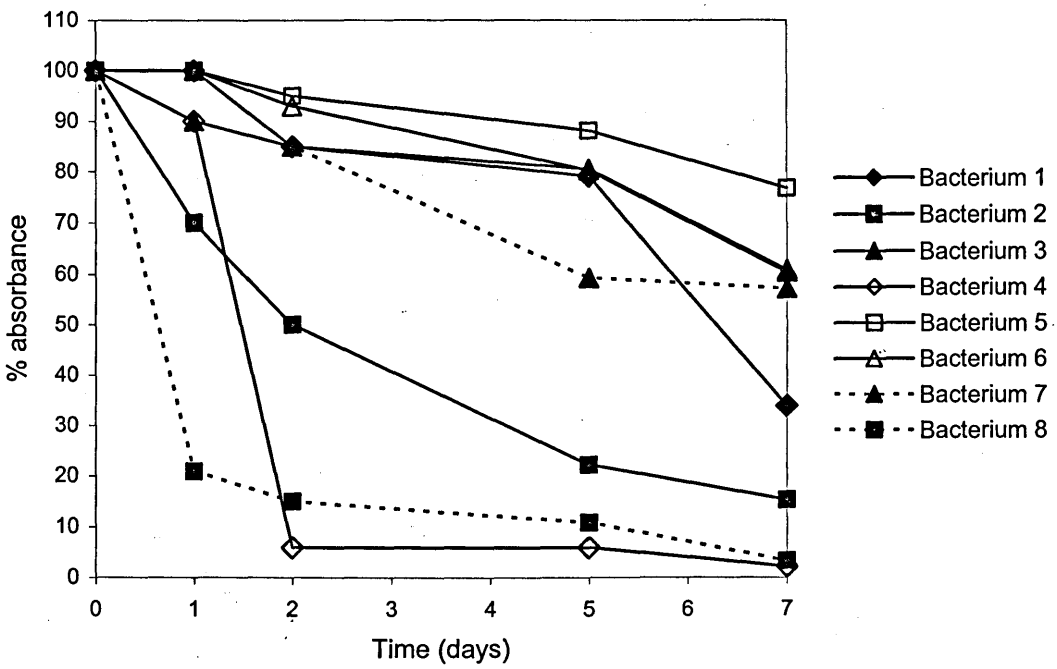


Figure 4.7 Colour removal of C.I. Acid Red 73 incubated under anaerobic conditions with eight different bacteria isolated from the activated sludge.

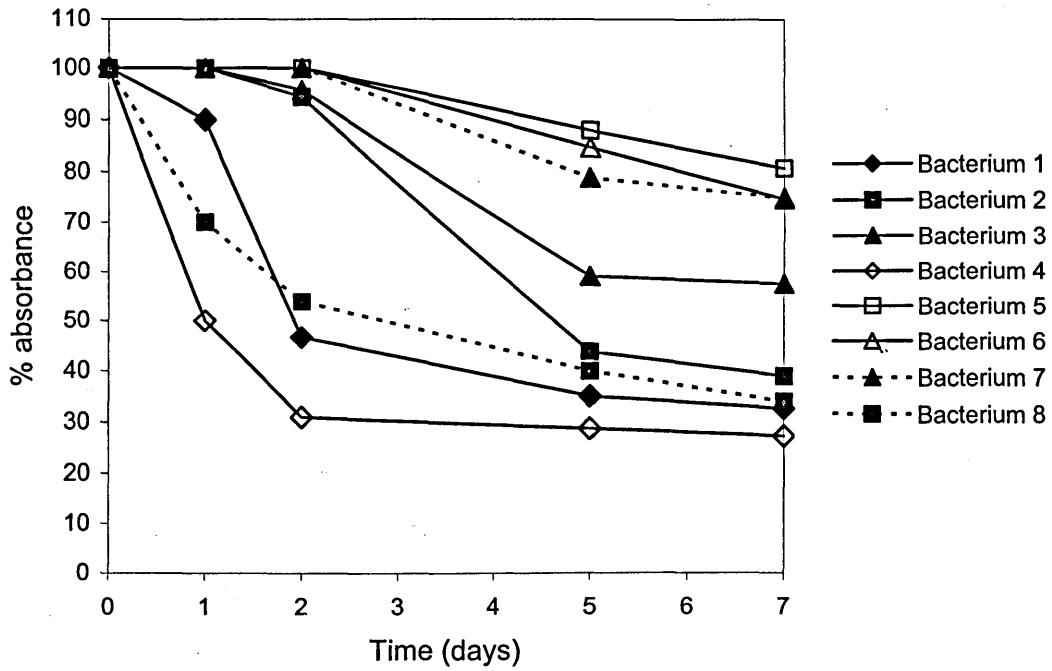


Figure 4.8 Colour removal of C.I. Reactive Black 5 incubated under anaerobic conditions with eight different bacteria isolated from the activated sludge.

For the bacteria 1, 2, 4 and 8, about 50 % decolourisation occurred between 24 to 48 hours (Fig. 4.8). According to Figure 4.7 and Table 4.5, the bacteria 1, 2, 4 and 8 seem to be the most efficient for decolourising C.I. Acid Red 73, as the pellets did not show much adsorption of the dye on the biomass.

Table 4.5 Pellet thickness and colour after centrifugation of the aliquots

Bacterium	C.I. Reactive Blue 19		C.I. Acid Red 73		C.I. Reactive Black 5	
	Thickness	Colour	Thickness	Colour	Thickness	Colour
1	Thin	Dark blue	Thin	Orange	Medium	White
2	Thin	Dark blue	Medium	Yellow-orange	Medium	Clear-brown
3	Thin	Dark blue	Thin	Red	Medium	Green
4	Thick	Dark blue	Medium	Yellow	Medium	Whitish
5	Thin	Dark blue	Medium	Red	Thin	Clear green
6	Thin	Red	Medium	Red	Medium	Red
7	Thick	Dark blue	Thick	Red	Medium	Green
8	Thick	Dark blue	Thin	Yellow	Medium	White

4.3.4 Identification of the isolated dye-decolourising bacteria using API 20NE

The isolated bacteria 1, 2, 4 and 8 were identified using the API 20NE test (Section 4.2.5) as *Pseudomonas* spp. (good identification to the genus level). It is important to ensure that the bacteria are not pathogenic and are not dangerous to handle, nevertheless, basic microbiologic safe handling (i.e. gloves, laboratory coat and sterile working conditions) is required at all time in any case.

The genus of these bacteria was confirmed by streaking them onto selective *Pseudomonas* medium. The isolated bacteria were cultured on two types of selective *Pseudomonas* agar, cetrimide - nalidixic (C-N) and cetrimide - fucidin-cephaloridine (C-F-C). The C-N agar is selective for *P. aeruginosa*, whereas the C-F-C agar is selective for any *Pseudomonas* species. The plates were incubated at 30°C for 48 hours.

The purpose of this was to confirm the identification of the bacteria as *Pseudomonas* spp. and ensure that none of them was *Pseudomonas aeruginosa*, a bacterium known to cause diseases such as respiratory tract

infections in susceptible individuals and is also known to be resistant to antibiotics.

Table 4.6 shows the visual observation made from the incubated plates for the four isolated bacteria. The observation of blue-green or brown pigmented colonies should indicate the presence of *P. aeruginosa* on the C-N agar plates.

According to Table 4.6, most of the plates showed bacterial growth apart for bacteria 4 and 8 on C-N agar. The growth of bacteria 1 and 2 were made of yellow-white colonies on the C-N agar, which means that they are not *P. aeruginosa*. Growth on the C-F-C agar of all the isolated bacteria indicates that they were all *Pseudomonas* spp. This extra experiment of culture of the isolated bacteria on selective *Pseudomonas* medium showed that none of the bacteria were pathogenic, and were therefore safe to be used to develop effluent treatment methods.

Table 4.6 Observation of colonies after 48h incubation of the agar plates at 30°C (with G, growth; NG, no growth and F, fluorescent colonies).

Bacterium	C-N *Pseudomonas* agar	C-F-C *Pseudomonas* agar
1	G*	G*
2	G*	G F
4	NG	G*
8	NG	G F

* Yellow-white colonies

4.4 Summary

This chapter has focused on the isolation of bacteria capable of removing the colour from dye solutions. Eight types of bacteria were isolated from a sample of Stevensons' activated sludge and were tested for colour removal over three dyes (C.I. Acid Red 73, C.I. Reactive Black 5 and C.I. Reactive Blue 19) under

aerobic and anaerobic conditions. Four out of the eight isolated bacteria were found able to decolourise dye samples. The highest decolourisation was achieved with anaerobic incubation of the azo dyes. These bacteria were selected, sub-cultured and stored for further study. The API 20NE test was used for their identification. The bacterial test revealed that they were all *Pseudomonas* spp. The culture of these bacteria on selective Pseudomonas C-N and C-F-C agar confirmed their genus identity as *Pseudomonas* spp and proved that none of them were *P. aeruginosa*, and hence, that none were pathogenic.

Further studies were then carried out to determine the optimum conditions for decolourisation of a selection of textile dye samples in order to design efficient textile effluent treatments (Chapter 5).

CHAPTER 5

DETERMINATION OF OPTIMAL CONDITIONS FOR DECOLOURISATION OF DYE SAMPLES BY STEVENSONS' BACTERIA

5.1 Introduction

Suspended microbial cultures in bioreactors are widely used for wastewater treatment. There is, however, a concern that high flow rates can cause biomass loss from the bioreactors resulting in a decrease of the treatment efficiency. Systems have therefore been developed to avoid loss by using fixed biomass processes. There is considerable interest in using immobilised microbial cells in industrial biotechnology. Advantages for fixed biomass systems, compared to the conventional biological treatments include higher volumetric load, increased process stability and more compact bioreactors (Lazarova *et al.*, 1994). Immobilisation is seen as a technique, which confines a catalytically active cell within a reactor system. The support must be sufficiently robust and stable to be retained by simple physical means within a reactor system where the bacterial cells are in contact with the substrate of nutrient (Portier, 1991). Many techniques for bacterial immobilisation have been studied, and adsorption to water-insoluble supports (organic or inorganic) has been the simplest immobilisation technique used so far. Many solid supports such as calcium alginate, stainless steel wire spheres, granular activated carbon, small cubes of wood and various polymers have been used (Bekers *et al.*, 2001; Guenette *et al.*, 1996; Henshaw *et al.*, 1999; Nishijima *et al.*, 1997; Rijnaarts *et al.*, 1995). These methods of immobilisation may involve adsorption, aggregate formation, covalent coupling or entrapment for the formation of biofilms on the support materials (Grunz *et al.*, 1999). It has been found that microorganisms may adhere to the surface reversibly for a short period of time, and become irreversible later (Portier, 1991).

Polymers such as polyethylene (PE), polystyrene (PS) or polypropylene (PP) beads or polyurethane (PU) foam are particularly advantageous support materials, because they are lower cost compared to some of the other materials and also because they are inert and tough with resistance to microorganisms. Some early investigations by Osserfort *et al.* (1966) have nevertheless established that PU for example can be biodegradable. Some microbial degradation of PU coatings by biofilms formed on surfaces such as prostheses or implants in the medical environment have also been reported (Costerton *et al.*, 1995; Marshall, 1992). According to Albertsson *et al.* (1987), UV light or oxidizing agents (e.g. UV sensitiser) can enhance biodegradation of PE, without them the natural biodegradation process would take more than ten years (Albertsson *et al*, 1988). Examples of the use of polymers as an immobilisation support for biomass is, however, well documented in the literature. Polymeric matrices have been reported as good immobilisation supports for fungi, used in the bioremediation of waste waters contaminated with heavy metals (Bai *et al.*, 2003; Degiorgi *et al.*, 2002; Ramelow *et al.*, 1996).

In the final part of this chapter, each type of polymer support was assessed for microbial immobilisation. The decolourisation of textile dyes by micro-organisms immobilised on the chosen polymer support was evaluated later (Chapter 7) to assess the feasibility of using an immobilised biomass continuous culture system, with integrated aerobic/anaerobic conditions, for the treatment of textile dye effluent.

5.2 Experimental

5.2.1 Chemicals and Equipment

Dextrin, acetate (from sodium acetate) and soluble wheat starch were provided by BDH. Ethanol was obtained from Sigma-Aldrich. They were chosen as four different types of carbon source for the experiments.

50 mL-vials with air-tight seals (Suba-Seal stoppers, Fisher Scientific, UK) were used for anaerobic culture; and 100 mL-flasks with foam cap for aerobic culture.

The polymers PE, PS, PP beads and PU foam were used to investigate the effect of polymer material on biomass support. The polymer beads were obtained from Sigma UK, and the PU foam was taken from foam caps used in the microbiology laboratory. The average diameter of polymer beads was 3 to 5 mm. The foam was roughly cut into pieces of approximately 2 to 3 cm diameter, randomly distributed in the flasks and vials for the batch culture. Its main characteristics were: average pore size (0.5 mm), density (32 g/L).

An electric autoclave, Omega™ media, Prestige™ Medical, was used to sterilise the media used in the experiments. An oxydoreduction probe (ORP), supplied by Russell pH Ltd, Scotland (K series combination pH and ORP electrodes) was used to measure the redox potential of effluent samples. Spectrophotometer, sonicator and incubator used were as described in section 4.2.1.

A vortex (WhirlMixer from Fisherbrand) was used to thoroughly mix test tubes in the protein assay procedure.

5.2.2 Textile dyes

Fourteen textile dyes were selected for the decolourisation experiments.

Remazol black B133% granulate,
Levafix golden-yellow EG,
Dianix red FB-E220,
Terasil rosa 2GLA,
granulate, Neutrilan black,
Diamond black PV200,
Acid Red 73,
Levafix brilliant red E-4B,
Evercion red E-4B,
Dianix yellow-brown HRSL-SE150,
Irgalan yellow 3RL KWL 250%
Lanaset blue 5G,
Diamond black PLC, C.I.
Everzol blue R special

They were provided by DyStar® UK. Their chemical structures are shown in Appendix B.

A stock solution was prepared for each dye (0.5 % w/v in distilled water) and was kept at room temperature. The maximum wavelength (λmax) of each dye was determined by running a wavelength scan of the dye prepared in distilled water at a concentration of 50 mg/L (Figs. 5.1 and 5.2). Table 5.1 indicates their colour index (C.I.) names and wavelength at λmax.

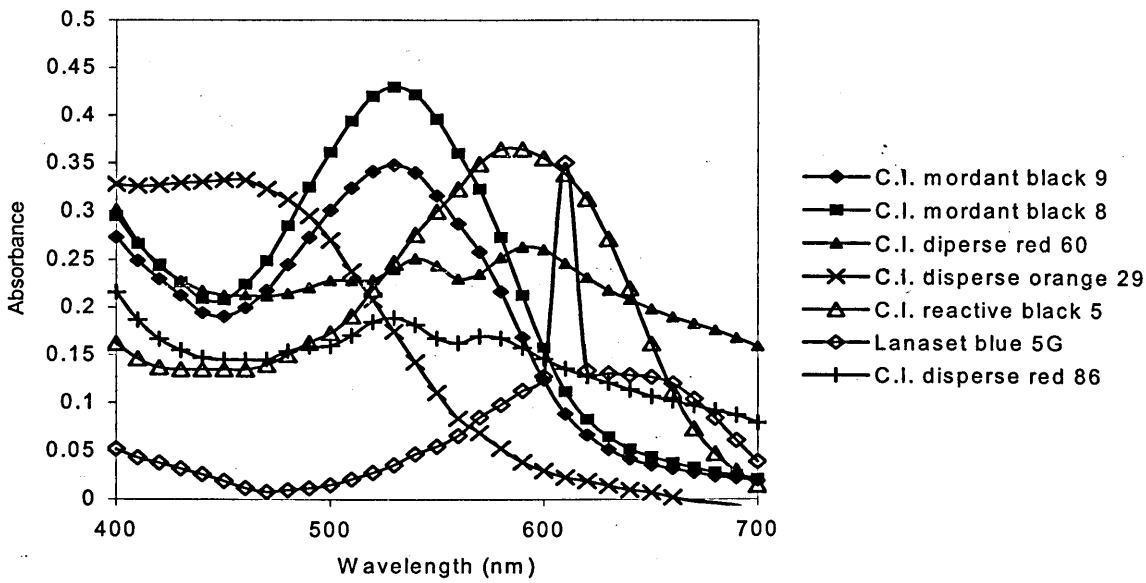


Figure 5.1 Wavelength scan of seven selected dyes.

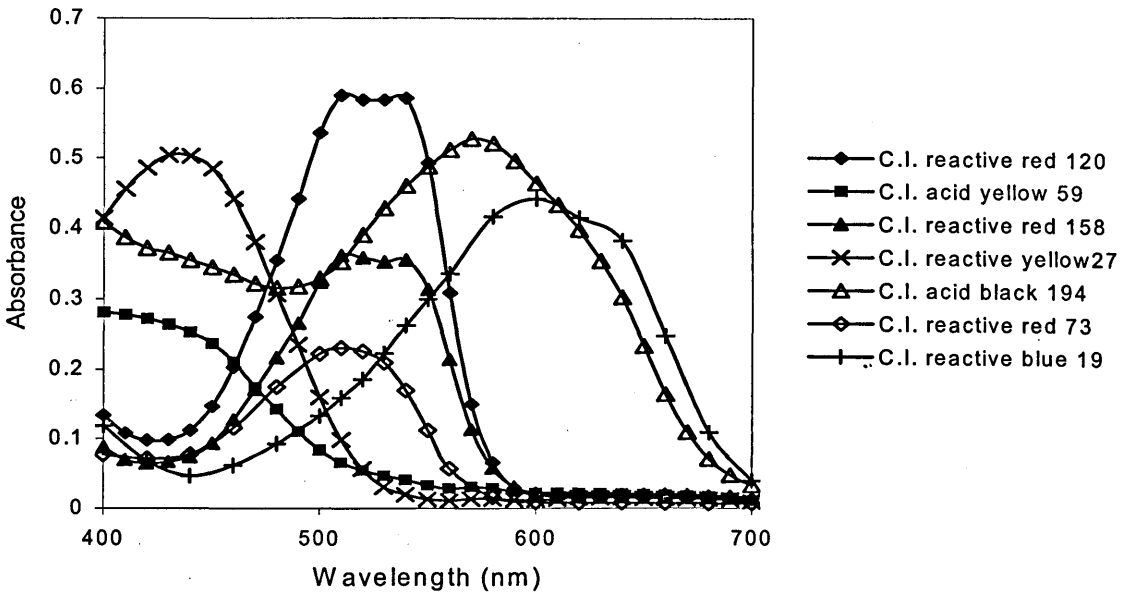


Figure 5.2 Wavelength scan of seven selected dyes.

Table 5.1 List of dyes with their wavelengths at peak absorbance (λmax).

Sample	Dye	Colour Index (C.I.)	Wavelength (nm) at λmax
1	Diamond black PV200	Mordant Black 9	530
2	Diamond black PLC	Mordant Black 8	530
3	Dianix red EFB	Disperse Red 60	590
4	Dianix goldbrown	Disperse orange 29	450
5	Remazol black B	Reactive Black 5	580
6	Lanaset blue 5G	Unknown	610
7	Terasil rosa 2GLA	Disperse Red 86	530
8	Evercion red E4B	Reactive Red 120	510
9	Irgalan gold 2GL	Acid Yellow 59	400
10	Levafix red E4BA	Reactive Red 158	510
11	Levafix yellow gold E-G	Reactive Yellow 27	430
12	Acid Red 73	Acid Red 73	520
13	Neutrilan black	Acid Black 194	570
14	Everzol blue R special	Reactive Blue 19	600

5.2.3 Decolourisation of the selected textile dyes by the isolated *Pseudomonas* spp.

Potential decolourisation of selected textile dyes by the mixed culture of *Pseudomonas* spp. was investigated under anaerobic and aerobic conditions. The bacteria were maintained on nutrient agar plates (at 30°C, aerobic conditions). Culture of the bacteria was performed in nutrient broth (Oxoid, UK) in culture flasks (incubated at 30°C for 24 hours, with shaking at 100 rpm) and was used as inoculum for the decolourisation experiments.

Each dye was prepared in nutrient broth at a concentration of 50 mg/L. The solutions and duplicates were autoclaved, and 100 mL portions were inoculated with 0.1 mL of the mixed culture of *Pseudomonas* spp. and incubated under anaerobic conditions and aerobic conditions at 25°C respectively. Glass vials (125 mL) fitted with air-tight seals (Suba-Seal stoppers, Fisher Scientific UK) were used to contain the dye samples under anaerobic culture conditions. Aerobic cultivation was carried out in 100 mL conical flasks fitted with foam caps. Cultures were incubated in the orbital incubator at 30°C with agitation at 100 rpm for 3 days. Samples (10 mL) were collected from the flasks and vials after 1 and 3 days incubation, and absorbances were measured at their λmax. The results, expressed as percentage absorbance and percentage decolourisation, were calculated from the absorbance values obtained at the time zero of incubation (A_0) and at the sampling time (A_t), according to the formulas:

$$\text{Percentage absorbance} = (A_t \times 100)/ A_0$$

$$\text{Percentage decolourisation} = 100 - [(A_t \times 100)/ A_0]$$

The percentage absorbance corresponds to the colour present in the sample or remaining after treatment.

5.2.4 Investigation of carbon sources for optimal colour removal

Four types of carbon sources: soluble wheat starch, sodium acetate, dextrin and ethanol are readily available, low-cost materials that they were chosen for the investigation of their effects on biomass growth and dye colour removal. Solutions of carbon source and azo dye for incubation were prepared with minimal salts medium and stock solutions of carbon source as described below.

Preparation of the minimal salts medium (MM) was carried out as follows:

Solution A: 2 mL/L of Vishniac and Santer solution (Table 5.2) and 2 g/L of KH_2PO_4, pH was adjusted to pH 8, and the solution made up to 900 mL.

Solution B: 3 g/L of NH_4Cl and 0.4 g/L of $MgSO_4.7H_2O$
The solution was made up to 100 mL.

Minimal salts medium (MM): Solutions A and Solution B were sterilised separately in an autoclave at 121°C for 15 minutes. The MM was then prepared by the addition of 900 mL of solution A (adjusted to pH 7.0) and 100 mL of solution B.

Table 5.2 The composition of Vishniac *et al.* (1957) trace element solution.

EDTA 50 g/L, $FeSO_4.7H_2O$ 5.0 g/L, $CaSO_4.5H_2O$ 1.57 g/L,	$ZnSO_4.7H_2O$ 2.2 g/L, $(NH_4)_6 Mo_7O_{24}.4H_2O$ 1.10 g/L, $CoCl.6H_2O$ 1.61 g/L,	$CaCl_2$ 5.54 g/L, $MnCl_2.4H_2O$ 5.06 g/L,
pH adjusted to pH 6.0 with KOH.		

Stock Solutions of each carbon source were prepared to contain 5 % of a carbon source and 0.5 g/L of C.I. Reactive Black 5. The sample solutions contained 90 mL of Minimal Salts Medium and 10 mL of the corresponding

stock solution. The final concentrations in the solution were 0.5 % of a carbon source and 50 mg/L C.I. Reactive Black 5. The pH of the solution was measured and recorded as pH 8.0.

Each solution of carbon source and azo dye was then inoculated with 0.1 mL of the mixed culture of *Pseudomonas* spp and incubated at 30°C under anaerobic and aerobic conditions for three days respectively. Control solutions were also prepared, these contained 0 % carbon source, 50 mg/L of C.I. Reactive Black 5 and were also inoculated with the mixed culture of bacteria. Samples taken after 1, 2 and 3 days were evaluated for decolourisation, pH, and redox potential.

5.2.5 Immobilisation of bacteria on polymer supports

Batch experiments for assessing bacterial immobilisation were carried out with four types of polymers: PU foam (with reticulated structure), PP, PE and PS beads. The polymers were washed three times with aqueous ethanol solution (10 % v/v) before use. One gram of each polymer was put in 50 mL of a mixed bacterial suspension of *Pseudomonas* spp. in nutrient broth (Oxoid) and incubated aerobically at 25°C with slow agitation (20 rpm). The mixed culture of *Pseudomonas* spp. was initially prepared by inoculating 50 mL of sterile nutrient broth with 0.1 mL of isolated cultures of each of the four dye-decolourising bacteria (Chapter 4).

Conical flasks containing 50 mL of the mixed culture of *Pseudomonas* spp. were used to carry out the experiments at 30°C under aerobic conditions, in slow agitation (20 rpm) for up to seven days. After 1, 2 and 7 days of incubation, the polymers were separated from the bacterial suspension and gently rinsed with 0.9 % sodium chloride. The polymers were then put in 20 mL distilled water and submitted to ultrasound using a sonicator for one minute in order to disrupt the bacterial aggregation formed on the polymers. Vigorous mixing was carried out using a vortex until a homogenous bacterial suspension was obtained. The amount of biomass bound to the polymers was determined by measuring the

protein concentration per gram of polymer. Cell lysis of the bacteria was performed in order to release their intracellular components, which include proteins. 0.5 mL was taken from each preparation and added to 0.5 mL of 2.0M NaOH. The samples were heated at 80°C for 5 minutes, the protein concentration was then measured, using the BIO-RAD protein assay (from BIO-RAD), according to the Bradford method (Bradford, 1976). The quantity of proteins measured should reflect the biomass concentration as the proportion of protein in dry weight bacterial biomass is relatively constant. The protein assay is a colorimetric measurement of protein samples using an UV-Visible spectrophotometer, as the BIO-RAD reagent (containing Coomassie blue dye) specifically binds to protein molecules, causing a shift in its λmax from 465 nm to 595 nm. The BIO-RAD reagent was diluted in distilled water (1 volume of dye: 4 volumes of distilled water). The solution was mixed then filtered through filter paper (Whatman N°1) and stored in a closed glass container at room temperature (for up to two weeks). Bovine serum albumin (BSA) supplied by Biochemika Fluka, was used as the standard protein for calibration. Several dilutions of the standard protein were prepared from a concentration of 0.2 to 1.4 mg protein / mL. Blanks, standards and triplicate samples were placed in clean, dry test tubes. A volume of 5 mL of diluted dye reagent was added to each tube, and then thoroughly mixed using a vortex. After 5 minutes, the absorbances were measured at 595 nm versus reagent blank.

5.3 Results

5.3.1 Decolourisation of selected textile dyes by *Pseudomonas* spp.

Fourteen selected textile dyes were individually incubated with the mixed culture of *Pseudomonas spp.* under anaerobic culture conditions for three days. Decolourisation was monitored after one and three days of incubation. Table 5.3 shows the percentage decolourisation of selected textile dyes with their standard deviation. The results indicate that most of the azo and anthraquinone dyes were partially decolourised (from 31 to 99 % colour removal after 3 days

incubation). Lanaset Blue 5G had the lowest percentage colour removal (10 %) after 3 days incubation.

Fig. 5.3 is a radar graph representing the percentage colour remaining in the 14 selected dyes after inoculation with the bacteria.

Table 5.3 Percentage decolourisation of selected textile dyes incubated with the mixed culture of *Pseudomonas spp.* under anaerobic culture conditions (results of triplicates are displayed as average of percentage decolourisation ± range).

Dye (C.I.)	Decolourisation (%) ± Range after one day incubation	Decolourisation (%) ± Range after three days incubation
Mordant Black 9	51 ± 7	60 ± 1
Mordant Black 8	43 ± 8	50 ± 1
Disperse Red 60	89 ± 6	95 ± 2
Disperse orange 29	69 ± 10	76 ± 2
Reactive Black 5	64 ± 18	83 ± 4
Lanaset blue 5G	0.5 ± 1	10 ± 3
Disperse Red 86	68 ± 16	76 ± 5
Reactive Red 120	84 ± 3	87 ± 1
Acid Yellow 59	41 ± 1	56 ± 1
Reactive Red 158	85 ± 3	91 ± 1
Reactive Yellow 27	60 ± 23	82 ± 2
Acid Red 73	98 ± 3	99 ± 2
Acid Black 194	31 ± 3	48 ± 11
Reactive Blue 19	30 ± 2	31 ± 4

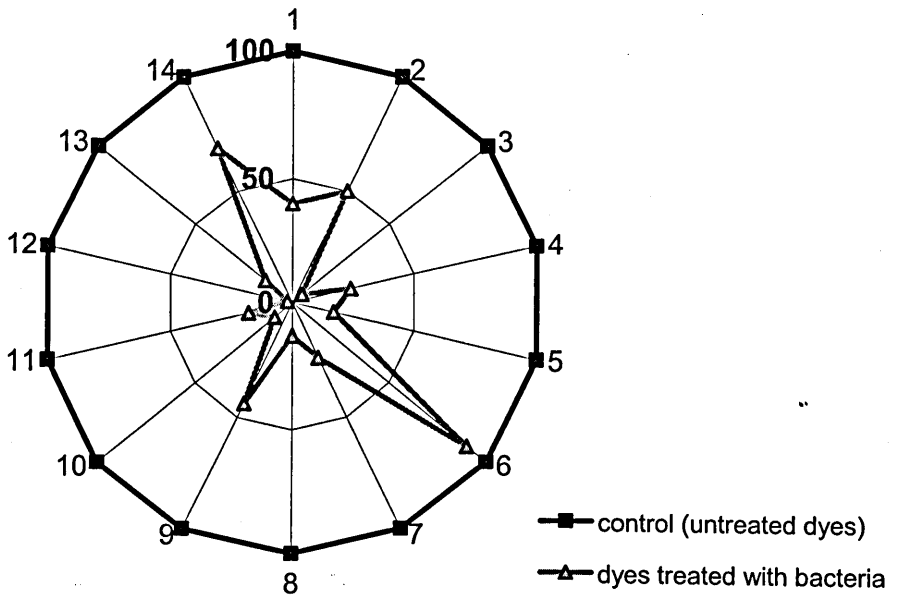


Figure 5.3 Radar graph representing colour measurements in percentage absorbances displayed from 0 to 100 % (from the centre of the circle) of the 14 dyes, before microbial treatment (time zero values) and after treatment, both under anaerobic conditions.

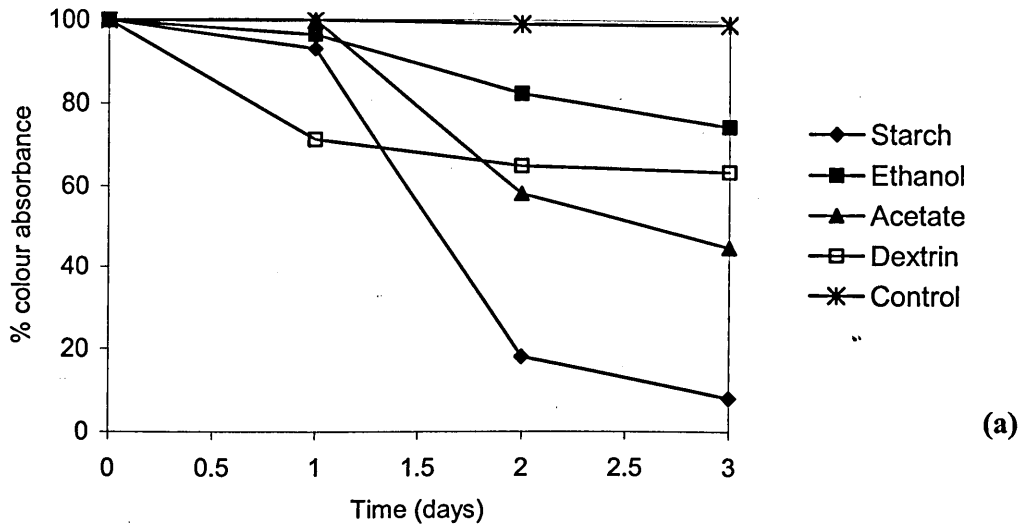
5.3.2 Investigation of carbon sources for optimal colour removal

The decolourisation experiments were carried out in nutrient broth (Oxoid), which is composed of lab lemco (beef extract) powder, yeast extract, peptone and sodium chloride (Section 4.2.2). The broth contains carbon, nitrogen and phosphorus in abundance as well as trace elements essential for bacterial growth. Such medium is only used in laboratory experiments, not in industrial-scale processes because of its cost. The use of alternative energy sources was therefore essential for the development of larger-scale systems.

Medium formulation is an important stage in the design of successful laboratory experiments or pilot-scale development. It is important to determine a suitable medium for a fermentation process, which aims to produce maximum yield of biomass, and which is also readily available, cheap and of a consistent quality.

Microbial biomass is composed of 50 % carbon, 10 % nitrogen and 3 % phosphorus; hence they need these components for their metabolism (Prescott *et al.*, 1999). The focus of this study was to identify a carbon source that would provide an adequate supply of energy for biomass maintenance. The results were used later in the development of a continuous culture system for effluent treatment, described in Chapter 7.

The results are shown in Figs. 5.4 - 5.6. Fig. 5.4 shows the effect of different types of carbon sources (soluble starch, ethanol, acetate and dextrin) on the decolourisation of C.I. Reactive Black 5 by *Pseudomonas* spp. grown on MM under (a) anaerobic and (b) aerobic conditions. Control samples (containing dye and MM only) were also inoculated with the bacteria and incubated under the same conditions. Among the four types of carbon source, the most effective decolourisation was achieved using soluble wheat starch. Results showed over 90 % decolourisation after three days of anaerobic incubation (Fig. 5.4a). Under aerobic conditions, however, the highest colour removal was 30 %, achieved with the acetate carbon source, after three days of incubation (Fig. 5.4b).



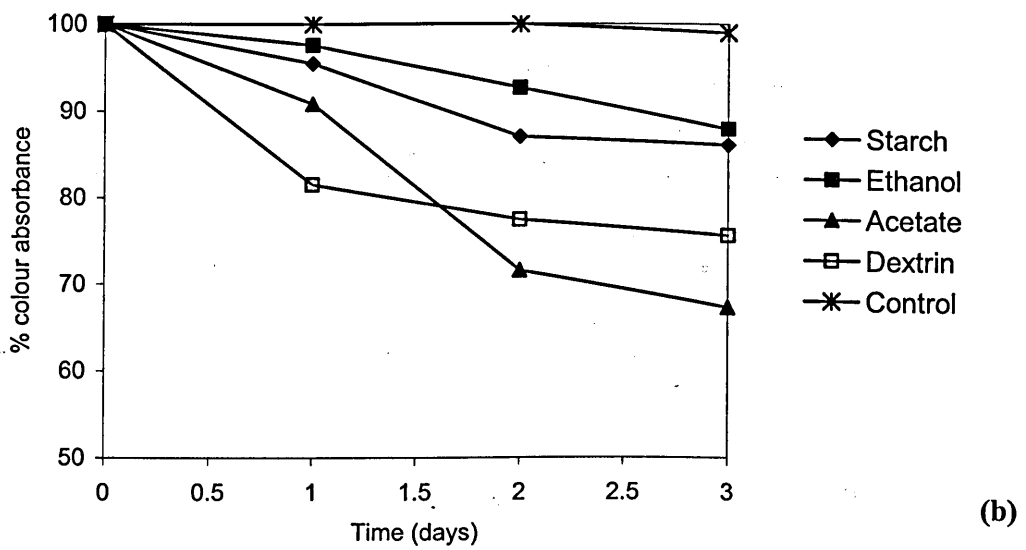
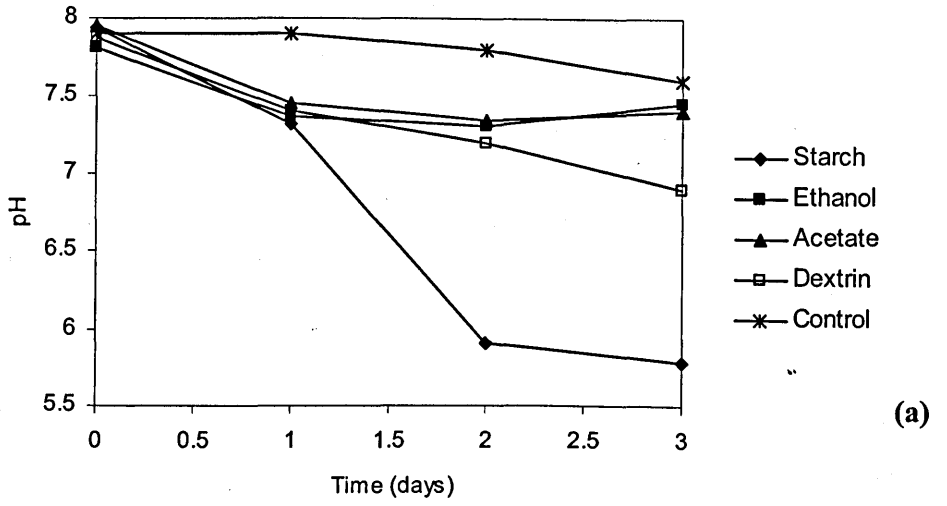


Figure 5.4 Effect of 5 % carbon source (soluble wheat starch, ethanol, acetate and dextrin) in MM with 50 mg/L of C.I. Reactive Black 5 (medium at pH 8.0) on colour removal by *Pseudomonas* spp. under (a) anaerobic and (b) aerobic conditions, at 30°C over three days. Control samples contained 50 mg/L of the dye in MM. Results are expressed as percentage colour absorbances.

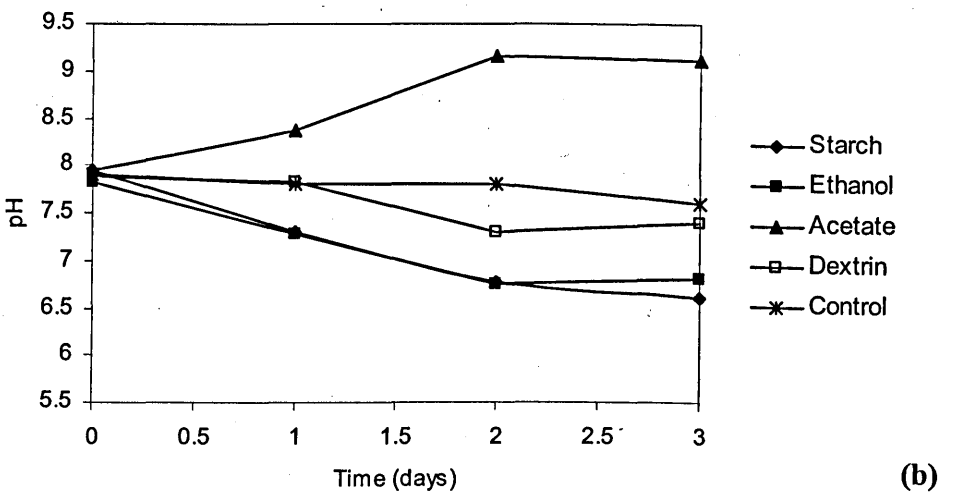
The controls show no decolourisation and hence indicate the importance of a carbon source in the dye degradation process. The contribution of the carbon source to biomass growth might play a role in the decolourisation mechanism. Anaerobic culture gave a more effective decolourisation of the starch sample than aerobic culture; soluble wheat starch was therefore chosen for formulation of the medium supporting biomass growth in further development of the effluent treatment system.

Figs. 5.5 and 5.6 show the pH and redox potential values measured over the incubation period. It is interesting to note that the pH of the sample containing starch decreased to a value of pH 5.8 after three days incubation in anaerobic batch culture (Fig. 5.5a). The acidification could be explained by fermentation in presence of starch leading to the formation of acid products such as acetic, succinic or lactic acids. The other carbon sources did not show any significant changes in pH values, which might be due to the fact that acetate and ethanol are non-fermentable carbon sources.

According to Carliell *et al.* (1995) and Beydilli *et al.* (1998), high dye colour removal corresponds to low redox potential of the dye sample. The redox potential values obtained (Fig. 5.6 a, b) did, however, not show any significant relation to the colour absorbances measured.

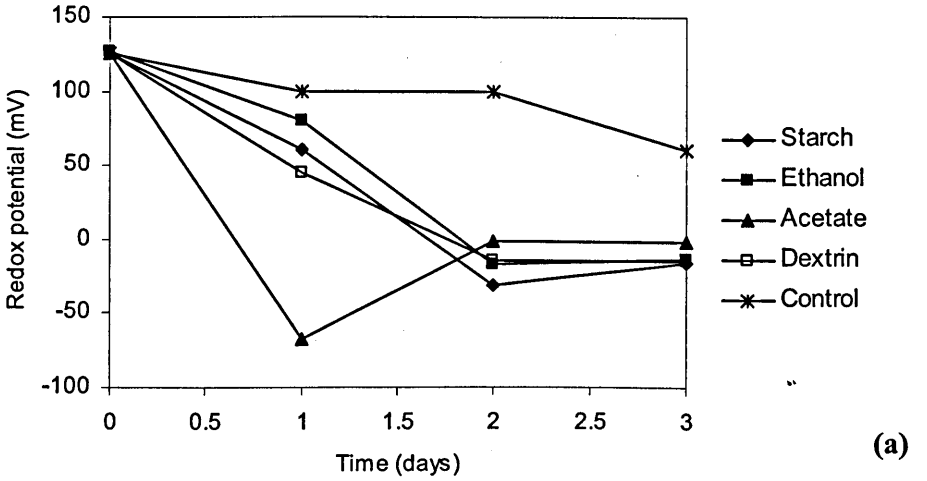


(a)

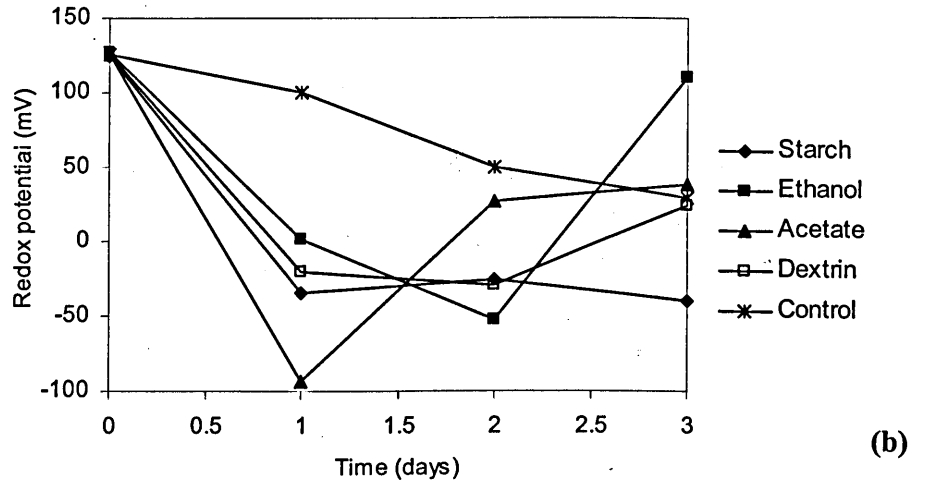


(b)

Figure 5.5 pH variations in dye/MM/carbon source samples inoculated with the bacteria and incubated over the 3 days at 30°C (a) under anaerobic and (b) under aerobic conditions. Controls did not contain any carbon source and were also inoculated with the bacteria.



(a)



(b)

Figure 5.6 Redox potentials (mV) of the dye/MM/carbon source samples inoculated with the bacteria and incubated over three days at 30°C (a) under anaerobic and (b) aerobic conditions. Controls did not contain any carbon source and were also inoculated with the bacteria.

5.3.3 Immobilisation of bacteria on polymer supports

Fig. 5.7 shows the quantity of biomass bound to each type of polymer after 1, 2 and 7 days of incubation in the mixed bacterial suspension. The immobilisation of the biomass on polyurethane foam was at a far higher density compared to the other polymers tested. Surface area and immobilisation support architecture were believed to be factors determining the extent of immobilisation, with relatively little biomass immobilised on the plastic beads, which were 2-4 mm diameter.

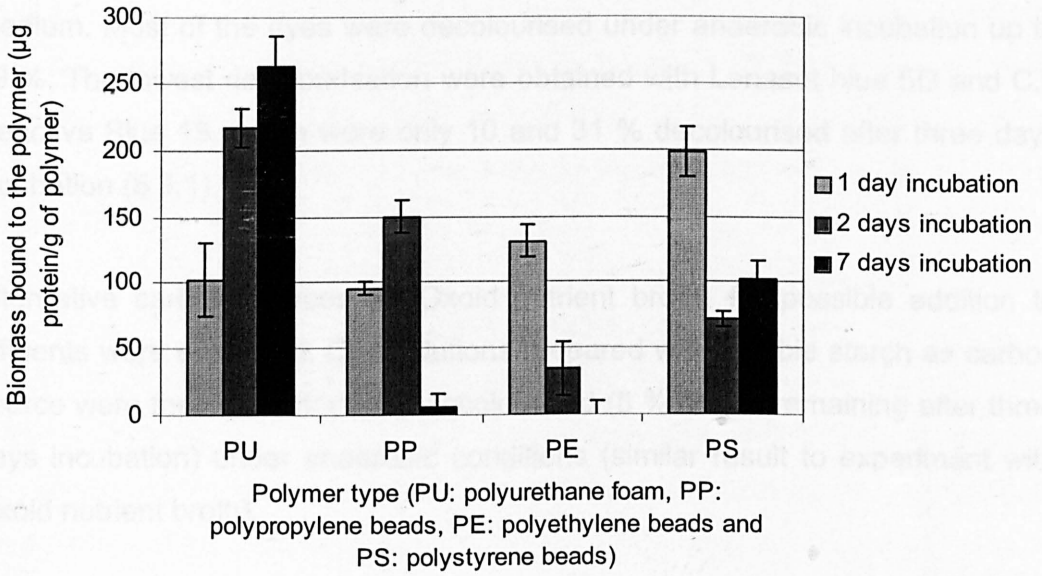


Figure 5.7 Evaluation of bacterial adhesion on different polymers (PU, PP, PE and PS) as µg protein / g of polymer. Triplicates were carried out (standard deviations are shown as error bars).

The results indicate that little biomass was bound to the other polymers (PP, PE and PS). Nevertheless, it is interesting to note that after seven days incubation, the polymer beads had less biomass attached to them than after one day. This might be explained by the conditions in which the experiments were carried out. Indeed, after seven days incubation in a batch culture, it was expected to find less living biomass because of the reduced carbon resources. The cells may die

on the support and get removed through shaking. Nevertheless, one would expect biomass to accumulate on the support through attachment of a few cells, which would grow and lead to colonisation of the support.

Dye decolourisation experiments using bacteria immobilised on PU foam are described in Chapter 7.

5.4 Conclusion

A range of selected textile dyes were used to investigate the level of dye decolourisation by the isolated *Pseudomonas* spp in Oxoid liquid nutrient medium. Most of the dyes were decolourised under anaerobic incubation up to 99 %. The lowest decolourisation were obtained with Lanaset blue 5G and C.I. Reactive Blue 19, which were only 10 and 31 % decolourised after three days incubation (5.3.1).

Alternative carbon sources (to Oxoid nutrient broth) for possible addition to effluents were evaluated. Dye solutions prepared with soluble starch as carbon source were the most effectively decolourised (8 % colour remaining after three days incubation) under anaerobic conditions (similar result to experiment with Oxoid nutrient broth).

The surface area of the PU foam was by far the highest compared to that of the other polymers studied and was the most efficient (surface area to concentration) for immobilising bacteria. These findings were used in further work on the development of a continuous culture system for the treatment of selected dye solutions and textile process effluents as described in Chapter 7.

CHAPTER 6

DECOLOURISATION OF SELECTED DYE SOLUTIONS USING LACCASE, AND TOXICITY MEASUREMENTS OF DYE DEGRADATION PRODUCTS

6.1 Introduction

The last decade has seen increased interest in the study of lignin white-rot basidiomycetes because of their ability to degrade recalcitrant organic pollutants (Field *et al.*, 1995; Vyas *et al.*, 1994). The white-rot fungus *Phanaerochaete chrysosporium* is well known for its capacity to degrade azo dyes and has been widely studied (Paszczynski *et al.*, 1995; Feng *et al.*, 1996 and Adosinda *et al.*, 2001). Other similar fungi include: *Trametes versicolor*, *Pleurotus* sp., *Bjerkandera* sp. and *Thelephora* sp. (Roy-Arcand *et al.*, 1991; Swamy *et al.*, 1999; Selvam *et al.*, 2002). A wide range of recalcitrant compounds can be degraded using enzymes, e.g. polycyclic aromatic hydrocarbons, chlorophenols, polychlorinated biphenyls and various azo, heterocyclic and polymeric dyes (Bogan *et al.*, 1996; Ricotta *et al.*, 1996; Xu, 1996; Wong *et al.*, 1999). The major enzymes associated with lignin degradation are laccase, lignin peroxidase and manganese peroxidase (Nyanhongo *et al.*, 2002).

Enzymatic treatments usually have low environmental impacts. They also have low energy requirements and are easy to operate over a wide range of pH, temperature and salinity. The potential use of oxidative enzymes (peroxidases and laccases) as biocatalysts in waste water treatment or waste remediation has been reported in the literature (Torres *et al.*, 2003; Duran *et al.*, 2000).

Laccases are multi-copper oxidases belonging to the family of the blue oxidase enzymes, catalysing the oxidation of a range of reducing compounds associated with the reduction of oxygen. The reason for the recent high interest in laccases is due to Bourbonnais *et al.* (1990) who found that laccases have a

broad substrate range including non-phenolic compounds in the presence of low molecular weight mediator compounds. Examples of mediators are 2,2'-azino-bis-(3-ethylthiazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT) (Li *et al.*, 1999), the presence of which increases the substrate specificity of laccases. According to Huttermann *et al.* (1988), one of the great advantages of these enzymes is their ability to detoxify phenolic compounds by means of polymerization reactions and cross coupling of pollutant phenols with naturally occurring phenols.

Laccases contain four copper atoms in the active site (Cu^{2+} in the resting state of the enzyme) that are classified in three groups according to their spectroscopic properties (Torres et *al.*, 2003):
T1: blue copper with an absorption band at 605nm detectable by electron paramagnetic resonance (EPR). T2: normal copper with no absorption band in the UV-Vis region and detectable by EPR. T3: coupled binuclear copper centre with an absorption band at 330nm, not detectable in EPR. Laccases usually contain: one T1, one T2 and two T3 copper ions. The groups T2 and T3 form a trinuclear copper cluster site, which gets involved in the binding of oxygen during its reduction to water (Klyachko *et al.*, 1992; Solomon *et al.*, 1996). The catalytic cycle proposed by Wesenberg *et al.* (2003) is illustrated in Fig. 6.1.

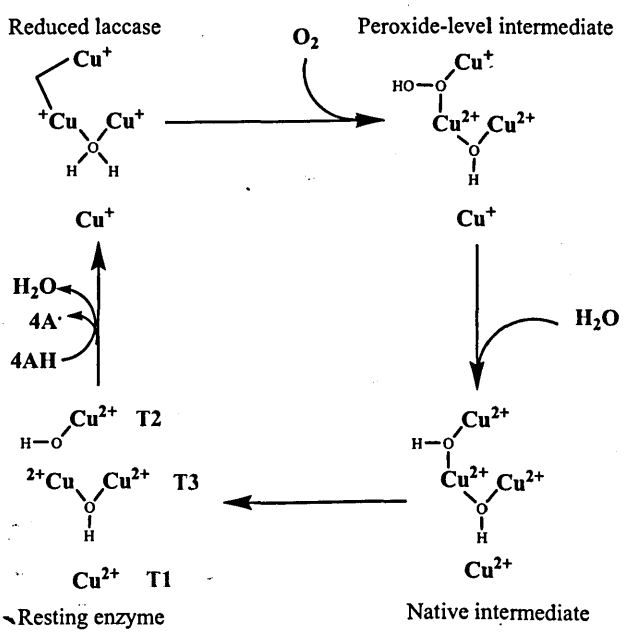


Figure 6.1 The catalytic cycle of laccases (Wesenberg *et al.*, 2003)

A similar model explaining the mechanism of the laccase catalytic cycle has been described by Torres *et al.* (2003). From the native form of the enzyme, a substrate molecule reduces the T1 site. A series of electron transfers and further reduction by other substrate molecules then occurs reducing all of the copper sites sequentially, resulting in a completely reduced state of the enzyme. The range of compounds oxidized by laccases can be increased by using mediators, which, when oxidized by laccases, produce very unstable and reactive cationic radicals, which in turn oxidize more complex compounds before returning to their initial state. The electrons taken by the laccase enzyme are then transferred back to oxygen to form water (McGuirl *et al.*, 1999; Wong *et al.*, 1999).

Toxicity is an issue to consider as many azo dyes, when treated using conventional biological treatments, may be broken down into toxic and or carcinogenic aromatic amines (Chung *et al.*, 1992). The need to find eco-efficient solutions to textile effluent treatments has led to the study of enzymes such as laccases for their potential to produce less toxic degradation products in effluent treatments. In this chapter, a laccase enzyme produced by *Trametes hirsuta* was assessed for its potential in dye decolourisation and detoxification.

6.2 Experimental

6.2.1 Materials and equipment

Laccase concentrates were kindly provided by KRKA, Novo mesto (Slovenia) and were produced by the micro-organism *Trametes hirsuta*, which was grown in a wheat bran based medium. KRKA concentrated the laccase using acetone precipitation and ultrafiltration, and then purified it according to Goncalves *et al.* (1996). The laccase crude extracts were kept at -20°C in the laboratory freezer as the enzyme rapidly looses its activity at room temperature. Aliquots were thawed and immediately used to carry out enzymatic experiments.

A succinate buffer solution was prepared from succinic acid and sodium succinate (supplied by Fisher Scientific Chemicals) to a concentration of 25 mM, and adjusted to various pH values ranging from 2.5 to 7. This buffer was chosen to carry out the enzymatic experiments following the method described by Niku-Paavola *et al.* (1988) with 2,2'-azinodi-(3-ethylbenzothiazoline-6-sulfuric acid) (ABTS) as substrate for the laccase enzyme and supplied by Sigma-Aldrich.

A Clifton unstirred water-bath was used to carry out enzymatic treatments at different temperatures. The colour of enzymatically decolourised dye samples were measured using a spectrophotometer as the one described in Section 4.2.1.

The toxicity of decolourised dye solutions was assessed using a SDI M500 Analyzer (Microtox® Rapid Toxicity Testing System from AZUR Environmental), illustrated in Fig. 6.2. Reagents were supplied with the instrument. These were reconstitution solution (specifically prepared non-toxic ultra pure water), diluent (specifically prepared non-toxic 2 % sodium chloride solution used for diluting the sample and the reagent, the marine bacterium in the reagent require osmotic protection provided by the diluent) and osmotic adjusting solution (OAS, specifically prepared non-toxic 22 % sodium chloride solution used to adjust the osmotic pressure of the sample to approximately 2 % sodium chloride). Glass cuvettes (3 mL) supplied by AZUR Environmental were used to contain the reagents and samples. The MicrotoxOmni™ computer software for Windows® 95/98/NT was used to analyse the samples absorbance values and calculate their toxicity. The software was also supplied by AZUR environmental.

Figure 6.2 Photograph of the SDI M500 Analyzer (AZUR Environmental).

6.2.2 Methods

6.2.2.1 Enzyme assay

Optimum temperature for the laccase activity was determined by measuring the decolourisation of an azo dye (C.I Reactive Black 5) at a fixed concentration of laccase (dilution factor 1000 of the crude extract) at selected temperatures ranging from 20 to 60°C. The dye was prepared at a concentration of 50 mg/L in 25 mM succinate buffer, pH 4.5. In each case, the dye solution was pre-incubated at the required temperature before starting the reaction by adding the enzyme. The contact time between laccase and the dye solution was 30 minutes. The percentage decolourisation of the dye sample was measured at λmax (580 nm) of C.I. Reactive Black 5.

The optimum pH for the laccase was determined by measuring the laccase activity at a fixed assay temperature of 30°C at various pH values varying between pH 2.5 and 7.0. The enzyme activity was measured using ABTS as a substrate according to the method published by Niku-Paavola *et al.* (1988). The measurement of enzymatic activity is based on the initial rate of the reaction. The reaction sample contained 2.3 mL of the suitably diluted enzyme in 25 mM succinate buffer at pH 4.5 and 0.7 mL ABTS. The reaction between laccase and ABTS (extinction coefficient ε = 29.3 $mM^{-1}.cm^{-1}$) create an increase in

absorbance at 436 nm, which was monitored for two minutes against the ABTS control (containing 2.3 mL succinate buffer pH 4.5 and 0.7 mL ABTS reagent only). The activity was expressed as nanokatals / mL (nkat/mL) and was calculated according to the formula:

Activity in Δnkat/mL (10^{-9} mole/second/mL) = (ΔAbs x cuvette volume x DF) / (ε x 60)

Where ΔAbs: absorbance increase within one minute;
cuvette volume: 3 mL;
DF: dilution factor;
ε, extinction coefficient of ABTS (29,300 M^{-1} cm^{-1} at 436 nm).

6.2.2.2 Enzymatic treatment of selected textile dyes

The results from the determination of optimum temperature and pH for laccase activity were used to carry out decolourisation experiments. The ability of the laccase to decolourise 14 selected textile dyes was investigated. The selected dyes were as described in Section 5.2.2. The reaction mixture was made of 100 mL succinate buffer (25 mM, pH 3.5) at 30°C containing the selected dye at a final concentration of 50 mg/L. The effect of the enzyme concentration on dye colour removal was assessed. Volumes of 100 µL, 40 µL and 20 µL of laccase were added to the dye/buffer solutions, corresponding to dilution factor 1000, 2500 and 5000 respectively. The effect of contact time with the enzyme on colour removal was also investigated. Laccase was diluted at DF1000 and was added to dye/buffer solutions (succinate buffer 25 mM, pH 3.5 and 50 mg/L of a dye). The reaction mixtures were kept at 30°C for eight hours. Absorbances were measured at the λmax of each dye. Colour measurements after treatment with the enzyme were expressed as percentage absorbance, as described in Section 5.2.3.

6.2.2.3 Determination of toxicity of dyes before and after treatments with *Pseudomonas* spp. and laccase using the Microtox® test system

The toxicity of dye samples before and after treatments was measured using the Microtox® test system. The principle of this system is based on the use of luminescent bacteria, specifically the strain *Vibrio fisheri*. Cell respiration is fundamental to cellular metabolism. Under normal growth conditions, *Vibrio fisheri* bacteria produce light as a by-product of their cellular respiration and any inhibition of cellular activity (toxicity) results in the decrease of cellular respiration and consequently to a decrease in luminescence. The tests were carried out using a Microtox Model 500 Analyzer, a laboratory-based temperature controlled photometer (15-27 °C), which maintains the luminescent bacteria and test samples at the appropriate test temperature. The sample toxicity is determined by measuring the effective concentration at which 50 % of the light is lost due to compound toxicity (EC_{50}). The percentage of light lost (if less than 50 %) can be taken as a toxicity result and is expressed as percentage effect. Results in Section 6.3.4 are expressed as percentage effect and correspond to the percentage of light lost measured from the marine bacteria and due to the sample toxicity.

Assay procedure: a vial of lyophilized bacteria (supplied by AZUR Environmental) was re-hydrated with 1 mL of the reconstitution solution to provide a ready-to-use suspension of micro-organisms. 0.5 mL of the suspension was added to 0.5 mL of diluent in a glass cuvette. Absorbance of the bacterial mixture was measured with the instrument and entered as the initial reading in the MicrotoxOmni™ computer software. The sample reagent contained 2.5 mL of sample (at pH 7.0 adjusted with NaOH or HCl) and 0.25 mL of OAS. A volume of 0.5 mL of the sample reagent was added to the previously prepared bacterial mixture. The cuvette was then mixed by shaking and left for 10 minutes. The absorbance of the bacterial mixture with sample was measured with the Microtox® instrument and entered as final reading in the computer software, which calculated toxicity results (expressed as the percentage of light reduction due to the sample).

Toxicity measurements of controls were also carried out, which results were used in the calculation of the dye sample toxicity results. The controls consisted of nutrient broth (sterile and 3-day incubated with 0.1 mL of the mixed culture of *Pseudomonas* spp.) and enzyme preparation (25 mM succinate buffer and laccase at DF 1000 in 25 mM succinate buffer). All the controls were adjusted to pH 7.0 with NaOH or HCl before the measurements were carried out. Toxicity measurements of the controls and samples were done in triplicate.

6.3 Results and discussion

6.3.1 Optimum temperature and pH for laccase activity

The activity of the laccase crude extract was measured as 939 nkat/mL.

The substrate used for the determination of optimum temperature for laccase was the azo dye C.I. Reactive Black 5. Results were shown as percentage colour removal, which relates to the laccase activity, i.e. high colour removal corresponds to high laccase activity. The activity of the laccase over a range of pH from 2.5 to 7 was measured and expressed as nkat/mL. Figs. 6.3 and 6.4 indicate that the optimal temperature and pH for the laccase enzyme were 30°C and pH 3.5 respectively.

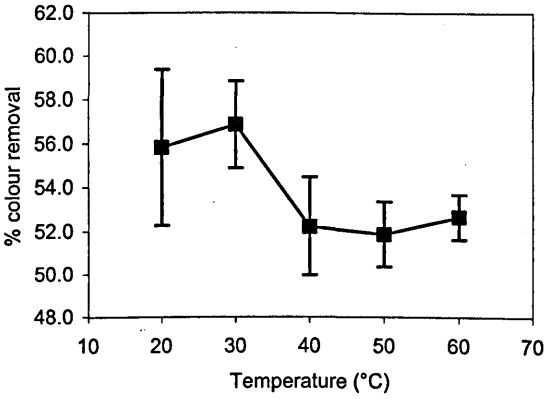


Figure 6.3 Determination of the optimum temperature for laccase by decolourising C.I. Reactive Black 5 (50 mg/L) in 25 mM succinate buffer (pH 4.5) with diluted laccase (dilution factor 1000). The percentage colour removal values were the average of duplicates and the ranges were shown as error bars.

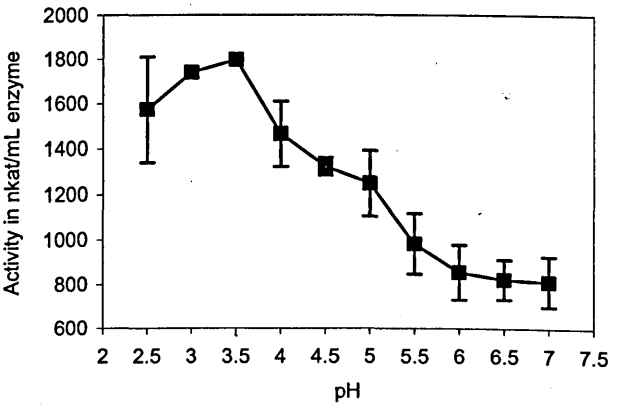


Figure 6.4 Determination of the optimum pH for laccase. ABTS was used as the substrate. The duplicate experiments were carried out at 30°C. The activity values were the average of duplicates and the ranges were expressed as error bars.

6.3.2 Decolourisation of selected dye solutions by laccase

Small amounts of laccase were required to achieve dye colour removal. The decolourisation experiments were carried out using the most efficient conditions for laccase activity previously determined (30°C and pH 3.5).

The effect of laccase concentration on dye decolourisation was studied using the enzyme diluted in succinate buffer solution (25 mM, pH 3.5) at three different dilution factors (DF): 5000, 2500 and 1000 (Fig. 6.5). According to Fig. 6.5, only four dyes out of the fourteen showed decolourisation after treatment with laccase (at DF1000), these were dye samples 1, 2, 6 and 14 (i.e. C.I. Mordant Black 9, C.I. Mordant Black 8, Lanaset blue 5G and C.I. Reactive Blue 19 respectively, see Table 5.1).

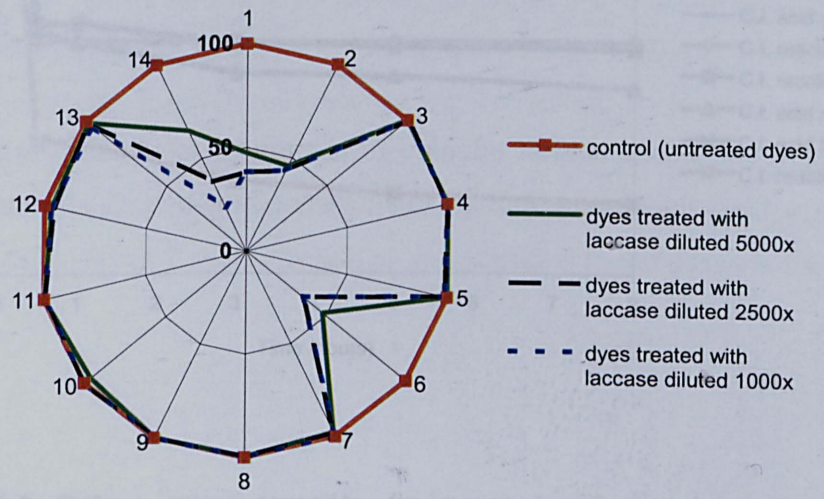


Figure 6.5 Radar graph showing colour measurements in percentage absorbance of 14 selected dye samples treated with laccase in 25 mM succinate buffer (pH 3.5) at 30°C for 30 minutes at different enzyme dilutions. The dyes samples were (1) C.I. Mordant Black 9, (2) C.I. Mordant Black 8, (3) C.I. Disperse Red 60, (4) C.I. disperse orange 29, (5) C.I. Reactive Black 5, (6) Lanaset blue 5G, (7) C.I. Disperse Red 86, (8) C.I. Reactive Red 120, (9) C.I. Acid Yellow 59, (10) C.I. Reactive Red 158, (11) C.I. Reactive Yellow 27, (12) C.I. Acid Red 73, (13) C.I. Acid Black 194 and (14) C.I. Reactive Blue 19 respectively.

The effect of contact time on dye decolourisation was also investigated using laccase at DF 1000 (Fig. 6.6). The same four dyes were decolourised after half an hour and the colour absorbance remained almost constant over the eight hours treatment. Dye sample 12 (C.I. Acid Red 73) showed a slow decrease in absorbance to 66 % after eight hours. The most effective decolourisation was achieved with dye 14 (C.I. Reactive Blue 19), with 12.5 % colour remaining at the end of the laccase treatment.

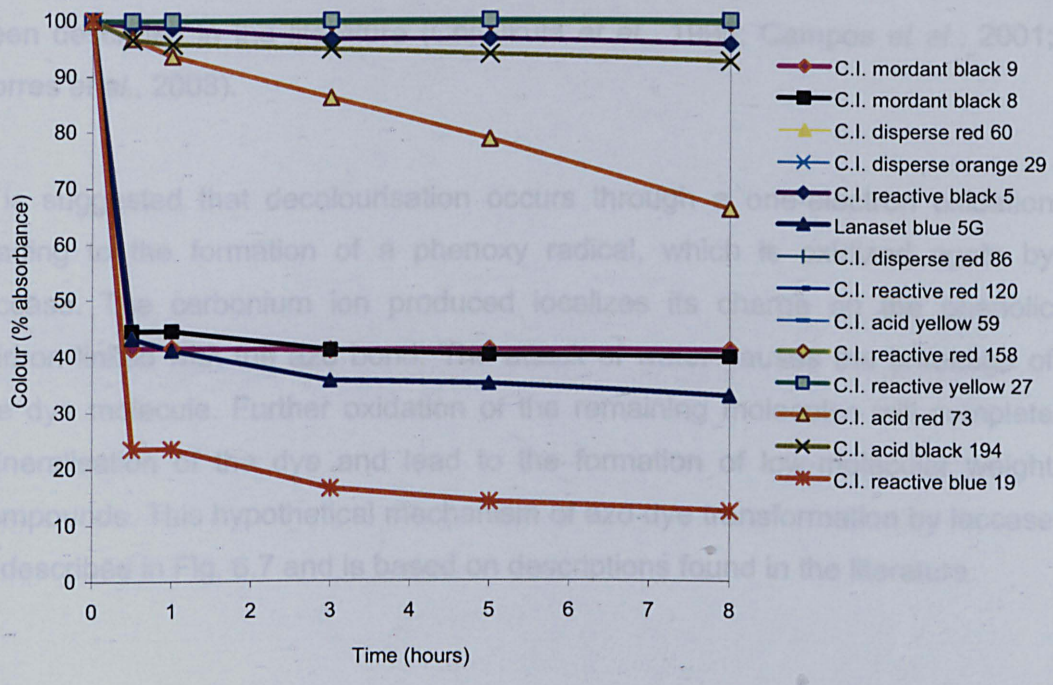


Figure 6.6 Colour measurements (in percentage absorbance) of the 14 selected dye samples treated with laccase at DF1000 in 25 mM succinate buffer (pH 3.5) at 30°C over 8 hours.

The lack of laccase catalysis on the ten other dyes could be explained by the fact that none of them are substrates for the enzyme. A case of inhibition of decolourisation has been reported by Soares *et al.* (2002), which was thought to be due to the presence of organic solvents (e.g. ethanol) responsible for changing the dye structure, for example azo to hydrazo form. This explanation is not possible here as the decolourisation experiments were carried out under

strictly similar conditions and did not involve any organic solvents. It is interesting to note that the four decolourised dyes were anthraquinone (C.I. Reactive Blue 19 and Lanaset blue 5G) and azo dyes (C.I. Mordant Black 8 and C.I. Mordant Black 9). The structure and or size of the dye molecules might have played a role in the laccase activity. However, these dyes did not decolourise completely (up to 76 % colour removed), which may be caused by the inhibition of enzyme activity by the dye-degradation products themselves.

Many suggestions for the mechanism of laccase oxidation of textile dyes have been described in the literature (Chivukula *et al.*, 1995; Campos *et al.*, 2001; Torres *et al.*, 2003).

It is suggested that decolourisation occurs through a one-electron oxidation leading to the formation of a phenoxy radical, which is oxidized again by laccase. The carbonium ion produced localizes its charge on the phenolic carbon linked with the azo bond. The attack of water causes the breakage of the dye molecule. Further oxidation of the remaining molecules will complete mineralisation of the dye and lead to the formation of low-molecular weight compounds. This hypothetical mechanism of azo dye transformation by laccase is described in Fig. 6.7 and is based on descriptions found in the literature.

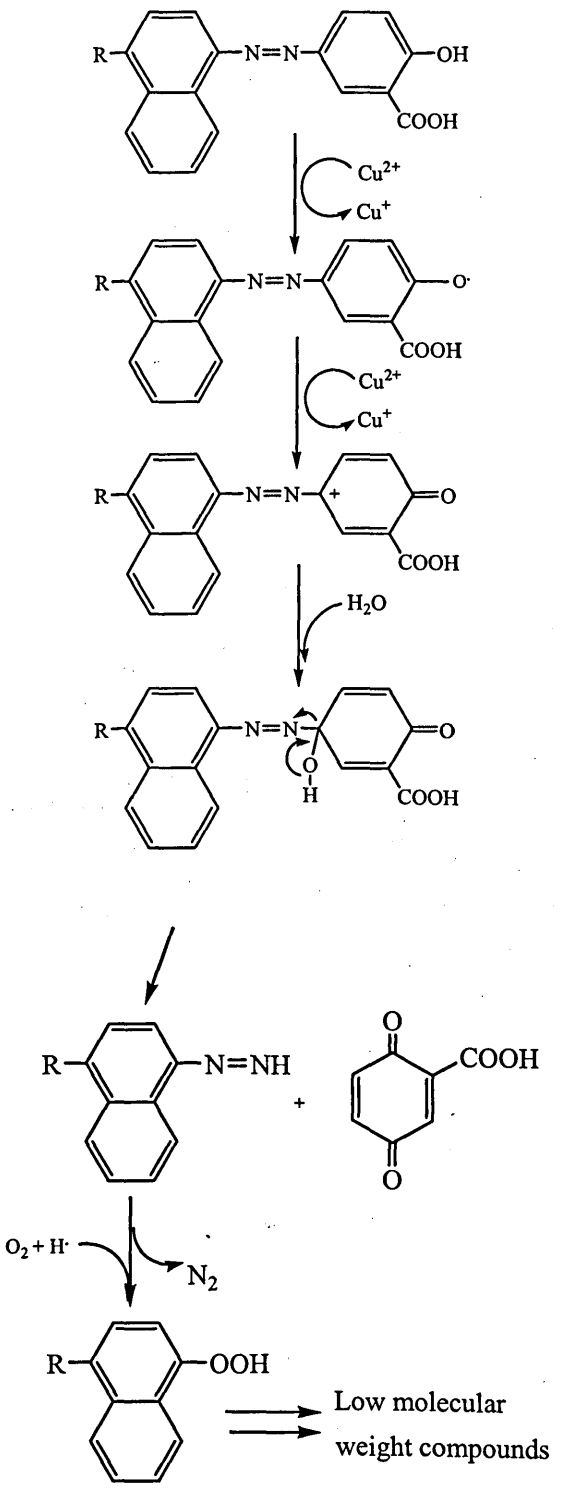


Figure 6.7 Suggested mechanism for azo dye transformation by laccase (based on Chivukula *et al.*, 1995 and Soares *et al.*, 2002)

6.3.3 Comparison with decolourisation of selected dye solutions by a mixed culture of *Pseudomonas* spp.

The results of decolourisation experiments carried out with bacteria and laccase were put together for comparison (Fig. 6.8). The bacterial system was able to decolourise a wide range of dyes (13 out of 14), with colour removal up to 98 % obtained with dye sample 12 (C.I. Acid Red 73). Only four dyes were decolourised with the laccase treatment, the maximum colour removal after 30 minutes of treatment was 76 %, obtained with dye sample 14 (C.I. Reactive Blue 19).

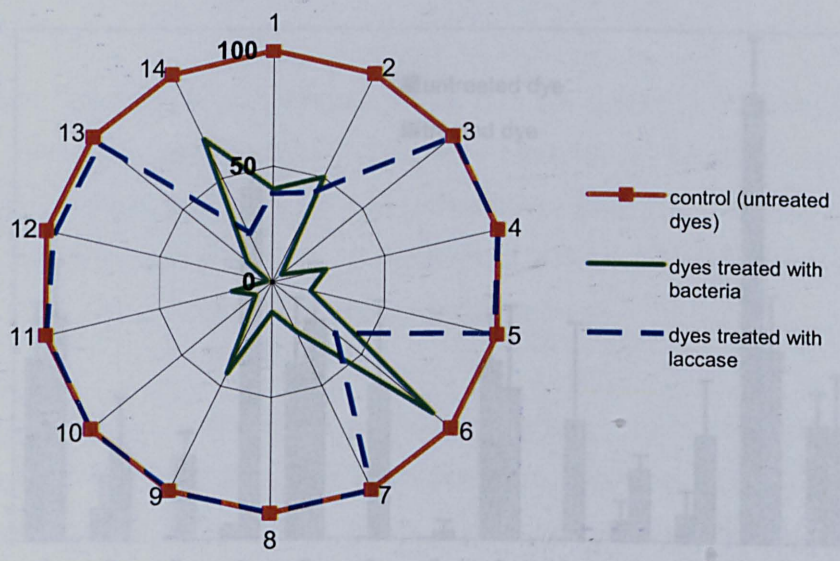


Figure 6.8 Radar graph comparing colour measurements (% colour) of 14 dye samples treated with a mixed culture of *Pseudomonas* spp. in nutrient broth medium (30°C, after 3 days incubation under anaerobic conditions) and treated with laccase (DF 1000, 30°C, in succinate buffer 25 mM, pH 3.5, for 30 minutes). The dyes samples were (1) C.I. Mordant Black 9, (2) C.I. Mordant Black 8, (3) C.I. Disperse Red 60, (4) C.I. disperse orange 29, (5) C.I. Reactive Black 5, (6) Lanaset blue 5G, (7) C.I. Disperse Red 86, (8) C.I. Reactive Red 120, (9) C.I. Acid Yellow 59, (10) C.I. Reactive Red 158, (11) C.I. Reactive Yellow 27, (12) C.I. Acid Red 73, (13) C.I. Acid Black 194 and (14) C.I. Reactive Blue 19 respectively.

6.3.4 Toxicity of dye solutions before and after treatment

Toxicity measurements of dye solutions before and after treatment with both systems (bacterial and enzymatic) were carried out for comparison, using the Microtox® test system. Toxicity of controls was measured and results were 8.5 % effect ± 1.8 for sterile nutrient broth, 23.2 % effect ± 2.2 for nutrient broth incubated with the bacteria, 3.5 % effect ± 1.3 for 25 mM succinate buffer and 7.7 % effect ± 2.0 for laccase at DF 1000 in 25 mM succinate buffer. These controls were taken into account in the calculations for toxicity levels of the dye samples. Figs. 6.9 and 6.10 show the results.

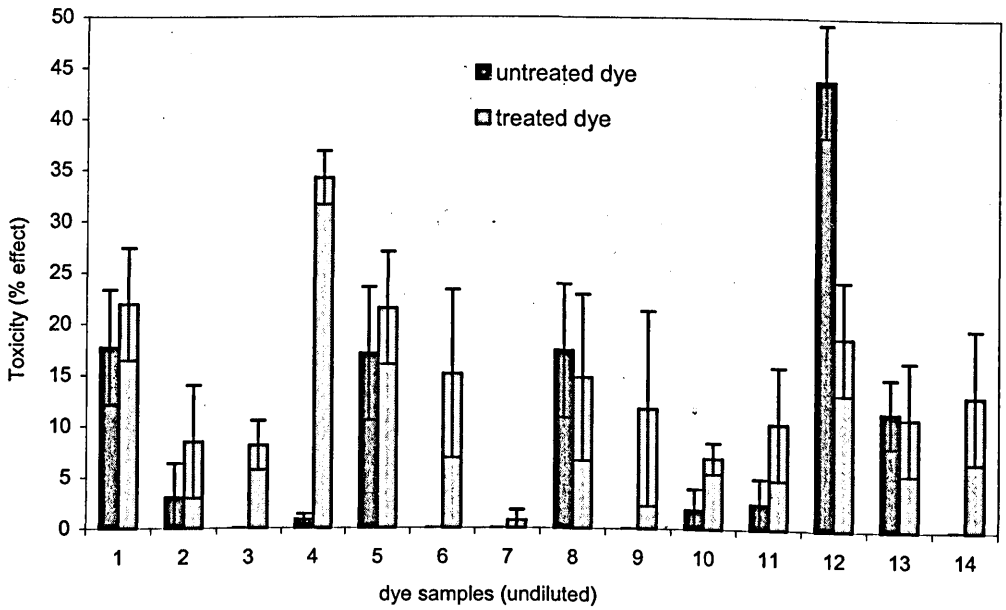


Figure 6.9 Toxicity measurements of the 14 dye samples before and after treatment with the mixed culture of *Pseudomonas* spp. in nutrient broth at 30°C under anaerobic conditions. Standard deviations are shown as error bars. The dye samples were (1) C.I. Mordant Black 9, (2) C.I. Mordant Black 8, (3) C.I. Disperse Red 60, (4) C.I. disperse orange 29, (5) C.I. Reactive Black 5, (6) Lanaset blue 5G, (7) C.I. Disperse Red 86, (8) C.I. Reactive Red 120, (9) C.I. Acid Yellow 59, (10) C.I. Reactive Red 158, (11) C.I. Reactive Yellow 27, (12) C.I. Acid Red 73, (13) C.I. Acid Black 194 and (14) C.I. Reactive Blue 19 respectively.

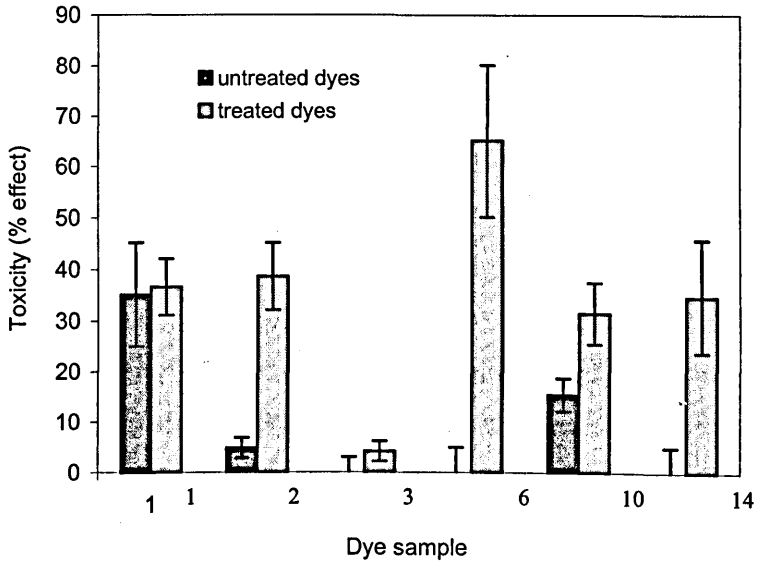


Figure 6.10 Toxicity measurements of the dye samples (1) C.I. Mordant Black 9, (2) C.I. Mordant Black 8, (3) C.I. Disperse Red 60, (3) Lanaset blue 5G, (10) C.I. Reactive Red 158 and (14) C.I. Reactive Blue 19, before and after treatment with laccase (DF1000, 30 minutes at 30°C in 25 mM succinate buffer, pH 3.5). Standard deviations are shown as error bars.

The toxicity levels are shown as percentage effect, which corresponds to the percentage of light reduction due to the sample, therefore, the more toxic the sample the higher the percentage effect. Fig. 6.9 shows that the toxicity of 11 of the 14 dyes increased after bacterial treatment under anaerobic conditions. The other three dye samples (8, 12 and 13) decreased their toxicity level after bacterial treatment, with samples 8, 12 and 13 showing 2.5 %, 25 % and 0.5 % toxicity decrease respectively. It is interesting to note that according to the results, five of the dye samples were not toxic in their natural state before decolourisation experiments: dyes 3, 6, 7, 9 and 14 (C.I. Disperse Red 60, Lanaset blue 5G, C.I. Disperse Red 86, C.I. Acid Yellow 59 and C.I. Reactive Blue 19 respectively).

The toxicity of dye degradation products after anaerobic treatments is well documented in the literature (Section 2.4.2). The reductive cleavage of the azo

bond in azo dyes gives rise to the formation of aromatic amines, which are known to be toxic. The highest increase in toxicity was observed with dye sample 4 (C.I. disperse orange 29), showing 33 % increase in percentage effect after bacterial treatment.

The toxicity level of samples decolourised by laccase treatment showed similar results. All the decolourised dyes (1, 2, 6 and 12) indicated increased toxicity after enzymatic treatment (Fig. 6.10). Dye samples 3 and 10, which were not decolourised at all by laccase treatment, were also monitored for toxicity as a comparison to the dyes that were decolourised by laccase (up to 76 %). Samples 3 and 10 showed slight increase in percentage effect after contact with the enzyme. Overall, enzymatic and bacterial treatments contributed to the increase in toxicity of the decolourised dye solutions, except for dyes 8, 12 and 13 (Fig. 6.9).

6.4 Conclusion

According to the results obtained, laccase treatment of selected dye samples showed rapid decolourisation of only four dyes up to 76 % colour absorbance, compared to biological treatment where thirteen dyes were decolourised over 3 days anaerobic incubation. Nevertheless, the decolourisation of dye samples occurred over 30 minutes and without the need of redox-mediators. Mediators have been widely used to promote enzymatic activity and expand the range of compounds oxidized by laccases. Some fungi have even been shown to produce their own low-molecular weight redox-mediators (Eggert *et al.*, 1996; Wong *et al.*, 1999). Decolourisation results might have been improved if mediators such as ABTS, HBT, or natural mediators such as veratryl alcohol, phenylacetic acid or tyrosine (Johannes *et al.*, 2000) were used in the reaction mixtures. Structure and dye concentration are also factors that might affect decolourisation rates.

The comparison between microbial and enzymatic systems was aiming at determining the potential use of laccase in textile effluent treatment and or

environmental detoxification. The present study suggests that laccase did not have wide substrate specificity (when used without mediators) and experiments needed to be carried out at specific pH and temperature (pH 3.5 and 30°C) for optimum enzymatic activity. The biological system for dye colour removal therefore seems to be the most attractive treatment method for decolourisation of the 14 selected model dyes. Toxicity of dye degradation products is, however, an important issue that still needs further study. Further experiments were carried out to assess the efficiency of biological treatment of dye effluents using a lab-scale continuous culture system with parameters comparable to those found in industrial effluent plants. This work is described in Chapter 7.

CHAPTER 7

DEVELOPMENT OF A LABORATORY-SCALE CONTINUOUS CULTURE SYSTEM FOR THE TREATMENT OF DYE SOLUTIONS AND TEXTILE EFFLUENT

7.1 Introduction

As described in previous chapters (Chapters 4 and 5), four strains of bacteria capable of decolourising a range of selected textile dyes were isolated from Stevensons' activated sludge and identified as *Pseudomonas* spp. Batch experiments were carried out in order to optimise decolourisation rates (Chapter 5). The optimum bacterial growth supports for decolourisation were determined as PU foam for immobilisation of bacteria and soluble wheat starch (0.5 % w/v) as a carbon source.

In this chapter, a laboratory-scale, continuous-culture 1 L- bioreactor containing the four isolated strains of *Pseudomonas* spp. was developed and used to evaluate the potential of the bacteria, immobilised on PU foam, for decolourising dyehouse effluent streams.

The continuous culture system was assessed for its performance through four different studies. The first experiment evaluated decolourisation of a chosen azo dye solution over a short period of time (up to three days). The second experiment was designed to investigate the optimum concentration of soluble wheat starch for dye colour removal. The third experiment assessed the continuous system over longer period of time (up to twelve days) operating at the optimum conditions investigated. Finally, a larger scale system with a 10 L bioreactor was set up and ran for twelve days, incorporating the findings described in Chapters 4-7. Decolourisation of real textile effluent was assessed.

7.2 Preliminary experiments using a laboratory-scale continuous culture system

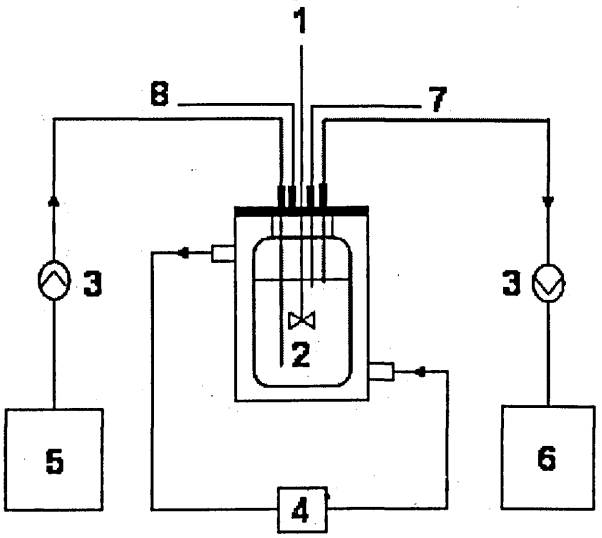
7.2.1 Materials and method

A cylindrical, glass reactor (from LH Fermentation) of capacity one litre was used for the continuous culture studies. The reactor was fitted with an integrated LH Fermentation 502D agitator (set at 10 rpm), used to homogenise the liquid in the reactor. The temperature in the bioreactor was maintained at constant 25°C with a Thermomix™ BU (4P B.Braun) water bath pump, which continuously circulated water in the external jacket of the bioreactor. Peristaltic pumps (Watson-Marlow) were used to supply the feed samples into the bottom of bioreactor (at a flow rate of 1 mL/min). In order to maintain a constant volume of bulk liquid within the reactor, the pump for the outflow was set at 1.5 mL/min pumping from the surface of the bulk medium (Fig.7.1).

As described in Section 5.2.5, PU foam was used as a biomass support in the reactor. It consisted of roughly cut pieces of approximately 2 to 3 cm diameter, randomly distributed in the reactor. Its main characteristics were 0.5 mm in average pore size and 3.2×10^{-2} kg/L in density.

The developed laboratory-scale, continuous culture system with a working volume of 1 litre was set up as shown in Fig. 7.1. The reactor was filled with PU foam (approximately 40g dry weight) and 1 L of nutrient broth. It was then inoculated with 10 mL of the mixed culture of *Pseudomonas* spp. and incubated at 25°C for 24 hours. The bacterial inoculum was prepared as described in Section 5.2.3. The agitator and pumps were then switched on so that the simulated dye effluent containing C.I. Acid Red 73 in minimal salts medium passed through the reactor. The flow rate was set such that the dilution rate and residence time were similar to those of the industrial-scale treatment plant in Stevensons, which has an average flow rate of 50 L/second. Therefore, the flow rate used was 1 mL/min.

As described in Chapter 5, soluble wheat starch (0.5 % w/v) was found to be the most efficient carbon source for dye colour removal, and was used as the nutrient source in the continuous culture.



1 Agitator
2 1L-Reactor containing PU foam
3 Pump set at 1mL / min
4 Water bath pump set at 25°C
5 Feed (influent)
6 Waste container (effluent)
7 Sampling tube
8 Air vent (with air filters)

Figure 7.1 Diagram of the continuous culture system with a 1 L-reactor.

Colour measurements were carried out to assess the efficiency of decolourisation in the reactor treatment system as described previously in Sections 4.2.4 and 5.2.3. Samples were taken from the bulk liquid within the reactor.

Biomass was measured by centrifugation of samples at 13,000 rpm for 5 minutes and re-suspension of the cell pellets to the same volume in distilled water with vigorous mixing using a vortex. The turbidity of the suspension was then determined spectrophotometrically at 540 nm against distilled water. Results are expressed as percentage absorbances and were calculated from the absorbance values obtained at the time zero of incubation (considered as 100 %) A_0 and at the sampling time A_t. The formula used for the calculation was similar to the one used for colour measurement, shown in Section 5.2.3.

The continuous fermentation systems were started after 1-day incubation of the reactor contents (inoculated polyurethane foam in nutrient broth). Three separate experiments for different simulated effluents were carried out.

Composition of the feed samples for the three experimental runs

(1) Minimal salts medium (MM) (as described in Section 5.2.4) and C.I. Acid Red 73 (at 50 mg/L concentration)

(2) MM, C.I. Acid Red 73 (at 50 mg/L concentration) and soluble wheat starch (0.5 % w/v)

(3) Real effluent taken from Stevensons balance tank

The pH of these media was adjusted to 7.0 with NaOH or HCl. Colour and biomass were measured from the samples, taken every day over the period of continuous operation. They were calculated and expressed as percentage absorbance as described in Section 5.2.3.

7.2.2 Results and discussion

Results show that the simulated effluent (dye and MM) and the real effluent have similar curves for colour and pH (Figs. 7.2 and 7.4). The increase in colour absorbance observed during the three days continuous culture was caused by the flow of dye coming into the bioreactor.

Conversely, the continuous culture, ran with the simulated effluent containing 0.5 % soluble starch (Dye + MM + 0.5 % starch), showed low colour absorbances, remaining under 3 % over 3 days of continuous culture. The corresponding biomass curve displayed some degree of constancy over 3 days, whereas the simulated dye and real effluent samples (without starch) caused a decrease in biomass (Fig. 7.2). The presence of starch seems to have promoted biomass growth and sustained it over the period of continuous culture operation.

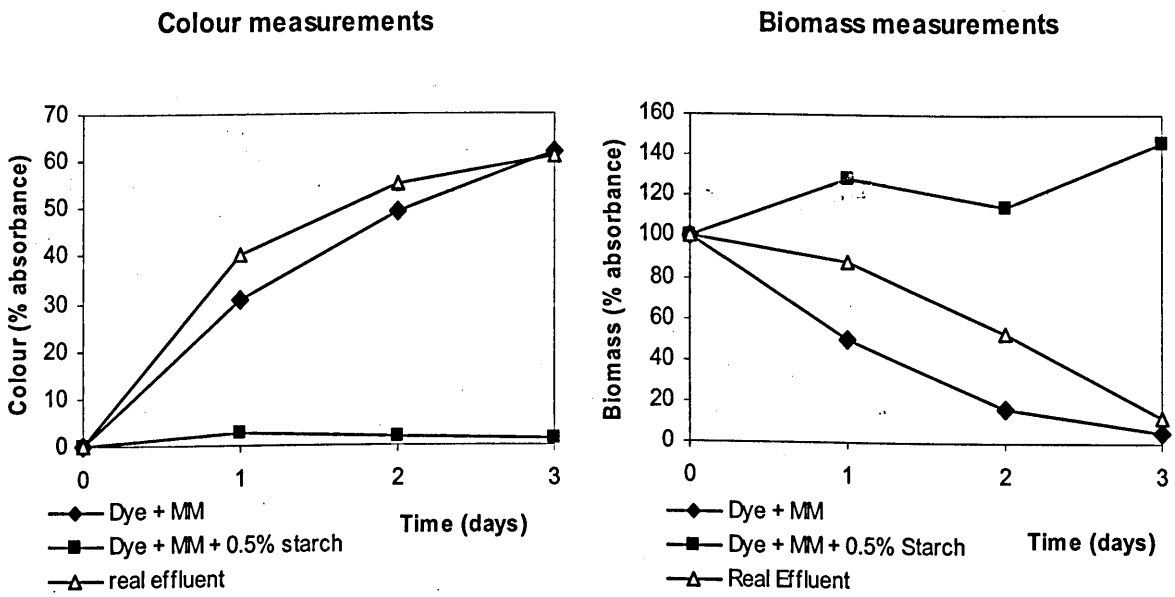


Figure 7.2 Changes in absorbance (percentage) for colour and biomass over three days of continuous culture.

Fig. 7.3 is a histogram comparing the percentage decolourisation between the three experiments. It shows that the dye solution containing soluble starch maintained a high percentage of colour removal, compared to the other samples. The carbon source seems to play an important role in the dye decolourisation mechanism by maintaining the biomass at a constant level.

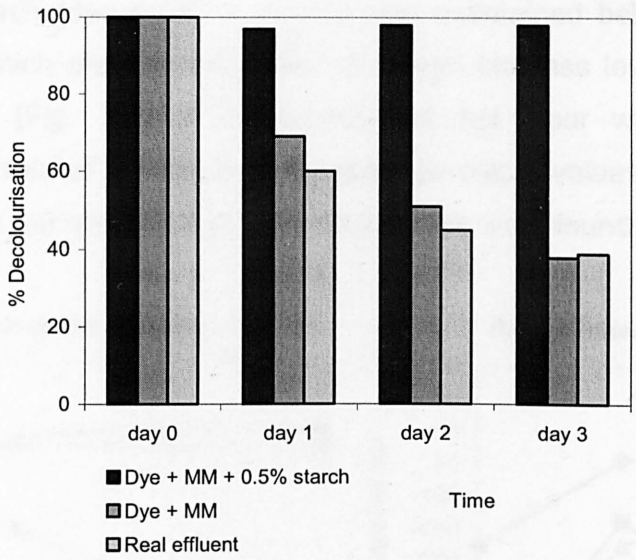


Figure 7.3 Comparison of percentage decolourisation between the three experimental runs over three-day continuous culture.

Fig. 7.4 shows the change of pH and redox potential over three days of continuous culture. It was found that the dye sample containing starch showed an acidification of the reactor medium during the continuous culture (pH 5.2 measured on the third day of operation). The presence of starch might have caused the acidification of the medium, which could be explained by the fermentation metabolism of bacteria other than *Pseudomonas*, as these are known to have a strictly respiratory metabolism and do not ferment. The continuous culture operations were not carried out in rigorous sterile conditions, the presence of bacteria other than the four strains of *Pseudomonas* spp. initially grown in the bioreactor is unavoidable. The competition between microbial species in such developed micro-niche is expected. Therefore, the use of a polymer matrix colonised by the *Pseudomonas* spp. is an advantage, creating a fixed biomass allowing re-growth of the required bacterial population in the presence of a carbon source.

The experiments carried out without starch (Dye + MM and real effluent) showed an increase in redox potentials over the three days of continuous culture, reaching approximately 0 mV. The redox potential for the experiment

containing starch (dye + MM + starch) was maintained below -250 mV after three days, which also corresponded to a high biomass level and low colour measurement (Fig. 7.2). It is nevertheless not clear whether low redox potentials are related to high biomass and low colour values. This was indeed not the case in Section 5.3.2, where redox values were found to be insignificant.

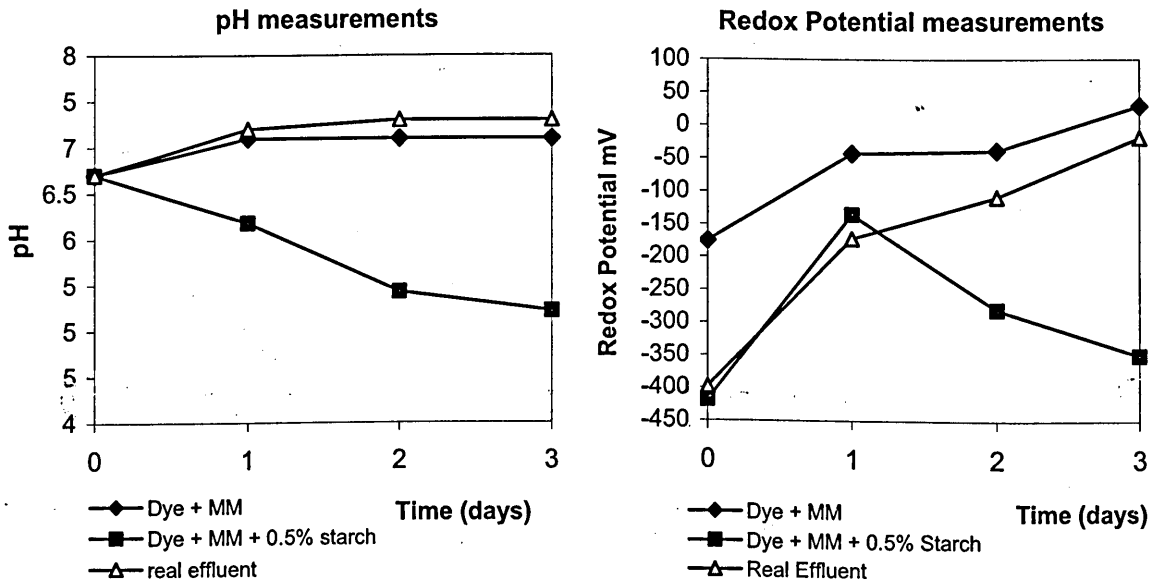


Figure 7.4 pH and redox potential measurements over three days of continuous culture.

Results of these preliminary experiments suggest that the use of soluble starch could provide more efficient colour removal. In the continuous culture experiment with real effluent, it was thought that organic substances contained in the effluent (contributing to high BOD_5 values) would have been sufficient to maintain biomass in the reactor. This was not the case, however, and the addition of a carbon source seems to be necessary.

Further experiments were carried out to determine the minimal starch concentration required to obtain maximum decolourisation. The effect of

prolonging the continuous culture period on decolourisation was also investigated (Sections 7.3 and 7.4).

7.3 Investigation of optimal starch concentration for colour removal in a laboratory-scale continuous culture system

7.3.1 Materials and methods

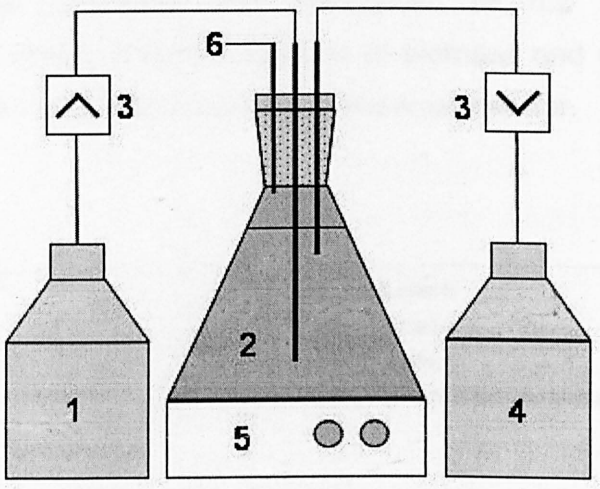
The effect of different concentrations of soluble starch on dye colour removal was evaluated over six days of continuous culture using a small scale continuous culture system (Fig. 7.5).

A conical flask containing approximately 5 g of PU foam roughly cut pieces (approximately 2 cm diameter), was filled with 200 mL of sterile nutrient broth and inoculated with a mixed culture of *Pseudomonas* spp. (inoculum prepared as described in Section 5.2.3). The flask was closed with a rubber cap, which was linked to feed and waste bottles through peristaltic pumps (set at 0.2 mL/min) using plastic tubing. The chosen flow rate is proportional to the one used with the 1 L-reactor, which was 1 mL/min (the working volume used here was 200 mL).

The continuous culture was carried out at room temperature and mixing was done using a magnetic stirer placed at the bottom of the flask underneath the foam. Dye solutions with three different concentrations of soluble starch were prepared: 0.5, 0.2 and 0.1 % (w/v), in order to investigate the effect of starch concentration on colour removal. A fourth experiment was carried out without starch. The dye solution was pumped through the reactor, which initially contained PU foam (5 g) with 200 mL of distilled water instead of the 200 mL inoculated nutrient broth.

Dye solution composition in the feed bottle for the four experiments:

1) MM with C.I. Acid Red 73 at 50 mg/L and 0.5 % soluble starch
2) MM with C.I. Acid Red 73 at 50 mg/L and 0.2 % soluble starch
3) MM with C.I. Acid Red 73 at 50 mg/L and 0.1 % soluble starch
4) MM with C.I. Acid Red 73 at 50 mg/L without soluble starch



1 Feed bottle (dye, MM, starch solution)
2 Conical flask containing PU foam in 200 mL bulk liquid
3 Peristaltic pumps
4 Decolourised dye solution
5 Magnetic stirrer
6 Air vent with filter

Figure 7.5 Bench-scale culture system using a 250 mL-conical flask as a small reactor packed with PU foam.

7.3.2. Results

Fig. 7.6 shows the effect of soluble starch concentration on colour removal during a 6-day continuous culture. It was found that 0.2 or 0.5 % starch gave similar colour absorbances. For a starch concentration of 0.1 %, the colour increase was more obvious after three days of continuous operation.

The optimal starch concentration seems to be between 0.1 and 0.2 %. The lowest starch concentration for optimum colour removal was 0.2 %, with less than 6 % absorbance after six days of continuous culture.

Previous experiments (Chapter 5) have demonstrated that soluble starch was the most effective carbon source for dye colour removal amongst other sources investigated. The lowest starch concentration for highest colour removal in these experimental conditions was determined as 0.2 %. The control experiment shows clearly that the absence of biomass and starch within the process results in a rapid colour increase in the small reactor.

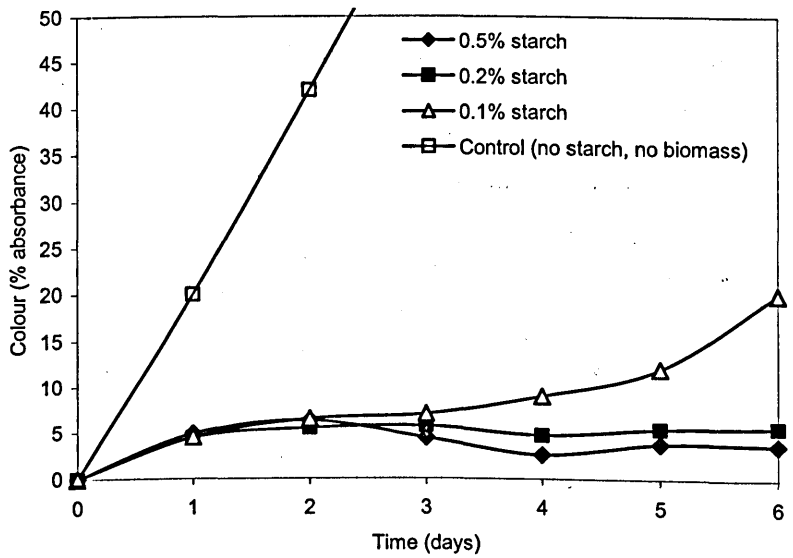


Figure 7.6 Effect of starch concentration on colour absorbances during a 6-day continuous culture. In the control experiment, the dye solution did not contain starch and the reactor did not contain any biomass.

7.4 Bioreactor studies on dye solutions and Stevensons' effluent over twelve days continuous culture

Dye solutions and real effluent samples were used to study the efficiency of the continuous culture system, described in Fig. 7.1, over a longer period of time

(twelve days). The aim of the experiment was to compare and evaluate the effect of the biomass supports (PU foam and soluble starch). The theoretical increase of dye colour in the reactor containing 1 L of distilled water, set up in a continuous process without biomass or foam, was also studied. The result was compared with the colour measurements of dye solutions and real effluent going through the reactor containing a mixed culture of the *Pseudomonas* spp.

7.4.1 Materials and methods

The laboratory continuous culture system described in Fig. 7.1 was used for the experiments. The bioreactor was filled with PU foam and 1 L of sterile nutrient broth, which was inoculated with 10 mL of a mixed culture of *Pseudomonas* spp. (inoculum prepared as described in Section 5.2.3). Temperature was set at 25°C and the bioreactor was left for 24 hours for the biomass to grow. Pumps were then set at 1 mL/min and the agitation rate was set at 10 rpm. Two experiments were carried out using the continuous culture system.

The first experiment used a dye solution (C.I. Acid Red 73) at 50 mg/L, prepared in MM and containing 0.2 % of soluble starch, which pH was adjusted to 7.0 with NaOH or HCl. The laboratory system was run for twelve days, with and without PU foam in the reactor, to assess the efficiency of the polymer biomass support (Fig. 7.8).

The second experiment involved the fermentation study of Stevensons' effluent. Samples from the balance tank were taken and used for the experiment (pH was adjusted to 7.0 with H_2SO_4). The wavelength for peak absorbance of the effluent was determined by wavelength scan. Fig. 7.7 shows λmax of the effluent sample at 510 nm. The reactor was packed with PU foam and 1 L nutrient broth inoculated with the bacteria. The continuous culture system was run for twelve days, first with raw effluent, then with effluent containing MM and 0.2 % soluble starch.

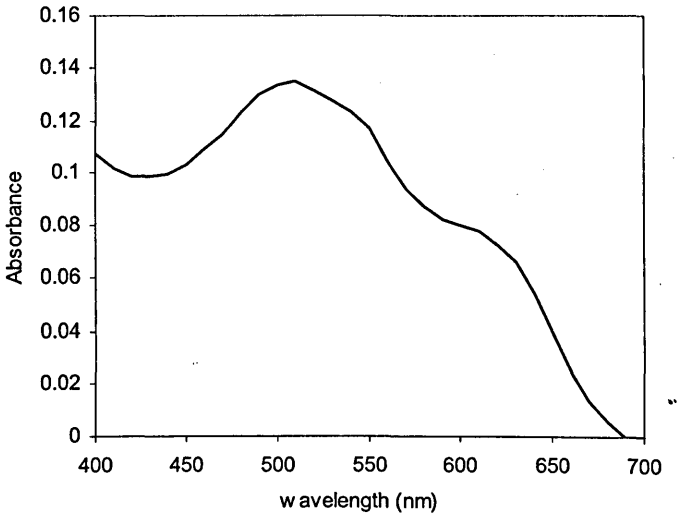


Figure 7.7 Wavelength scan of Stevensons' raw effluent.

Theoretical colour curve

The colour increase in a bioreactor containing initially 1 L of distilled water, without PU foam and bacteria was calculated. A coloured solution, such as C.I. Acid Red 73 prepared in distilled water at 50 mg/L was used as the feed sample. The flow rate for the considered system was 1 mL/min and the mixing in the reactor was considered as homogenous. The differential equation for absorbance increase over time in the reactor is shown to be of a single order, open loop control system (equation (5)).

Considering the time period t = 0...t, and the process system described in Fig. 7.1 (with the reactor filled with 1 L of distilled water at t = 0). The increase of colour in the reactor is st, whereas the amount of colour removed from the reactor at the same time is given by the integration:

$$s\int_0^t q(t)dt \qquad (1)$$

Therefore the quantity of colour in the reactor at the time (t) is:

$$q(t) = st - s\int_0^t q(t)dt \qquad (2)$$

This integral equation can be rewritten in derivative form:

$$q'(t) = s - sq(t) \qquad (3)$$

The differential equation for concentration of colour in the reactor can be written as:

$$q'(t) + \frac{s}{V}q(t) = \frac{s}{V} \qquad (4)$$

Equation (4) represents a family of linear differential equations with constant coefficients. The solution of such equation is given by:

$$q(t) = 1 - e^{-\frac{s}{V}t} \qquad (5)$$

Solution (5) takes into account the initial conditions of the process, i.e. there was no colour at the time t = 0, q(0) = 0. If the values for s and V are substituted into (5), the following equation is obtained:

$$q(t) = 1 - e^{-1.44t} \qquad (6)$$

Notations: $q(t)$, quantity of colour in the reactor, function of time (in days); V, Volume (1 L); s, flow rate (1 mL/min); q(t) = q(t)/V, concentration of colour in the reactor, function of time.

The curve for equation (6) was plotted and compared with results from the other experiments (Figs. 7.8 and 7.9).

7.4.2 Results

Fig. 7.8 shows three colour absorbance curves. The theoretical colour curve corresponds to the increase of colour that would occur in the reactor under the conditions described earlier (Section 7.4.1). It is interesting to note that colour rapidly reaches 100 % after approximately three days of experimental run.
In the absence of PU foam in the reactor, the colour absorbances tend to increase with a faster rate than the experiment with support, reaching 37 % colour absorbency after twelve days continuous run. When PU foam was present in the reactor, colour measurements showed low absorbances over the experimental period (7 % colour remaining on the twelfth day). Compared to the theoretical colour curve, both experimental results kept low colour absorbances in the reactor (below 37 % over the experimental period), showing the efficiency of the treatment system.

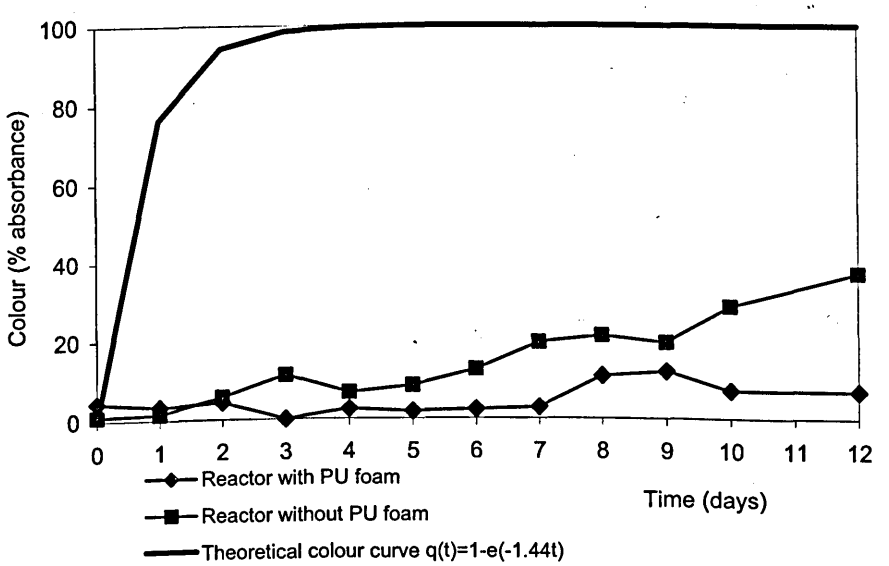


Figure 7.8 Decolourisation study of a dye solution (C.I. Acid Red 73 in MM + 0.2% soluble starch) in the reactor culture system filled with or without PU foam. Results were compared with the theoretical colour increase curve.

The second experiment involved the decolourisation study of Stevensons' effluent samples (Fig. 7.9). The aim of the experiment was to assess the importance of nutrients and PU foam as biomass support in the reactor and their effect on dye decolourisation. There was a net difference between raw effluent and effluent with nutrients. The raw effluent showed a fast increase in colour absorbances in the bioreactor compared to the experiment with effluent containing soluble starch. The addition of nutrients to the effluent promoted colour removal over twelve days of continuous culture operation (Fig.7.9). The effluent was completely decolourised over six days and slightly increased in colour thereafter, with 20 % colour remaining on the twelfth-day of the experiment.

The theoretical colour curve was also plotted for comparison. It is interesting to observe that results from the experiment with raw effluent showed a similar curve. It is possible that raw effluent from Stevensons' balance tank might not contain sufficient carbon sources that can be metabolised by the mixed culture of bacteria, and hence might not be able to promote sufficient growth for decolourisation to occur. This could explain the rapid increase of colour in the reactor when raw effluent was used as feed sample, despite the presence of PU foam in the reactor. Nevertheless, the addition of soluble starch showed that a carbon source is essential in the performance of the laboratory-scale continuous culture system.

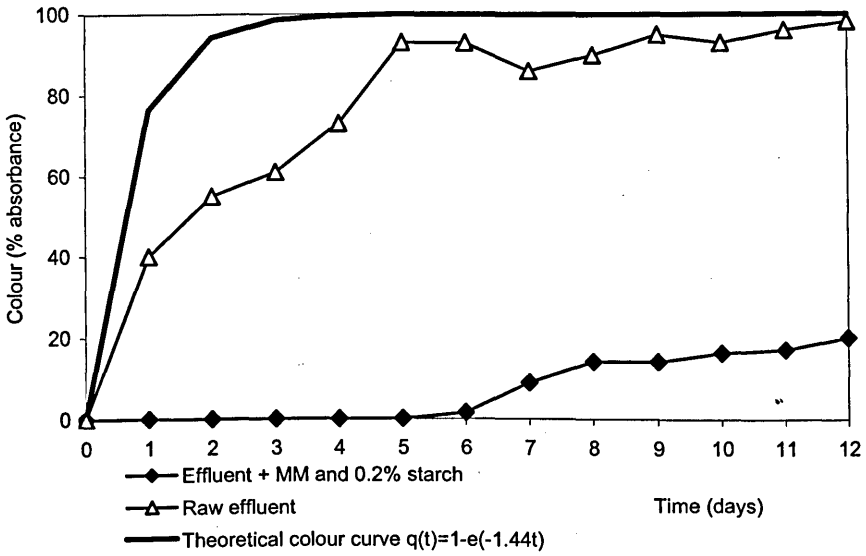


Figure 7.9 Decolourisation study of real effluent samples (adjusted to pH 7.0), raw and containing MM + 0.2 % soluble starch, going through a reactor filled with PU foam and bacteria. The colour measurements were compared to the theoretical colour curve.

7.5 Ten-fold scale-up of the continuous culture system

The aim of this part of the project was to develop a larger-scale laboratory process and assess its performance for textile effluent treatment. The concentration of aromatic amines in decolourised effluent samples was measured to assess the dye reduction metabolism of the biomass throughout the laboratory process.

Dyeing experiments were carried out using the treated effluent to compare and assess the quality of dyed fabrics with that of those dyed using normal supply water.

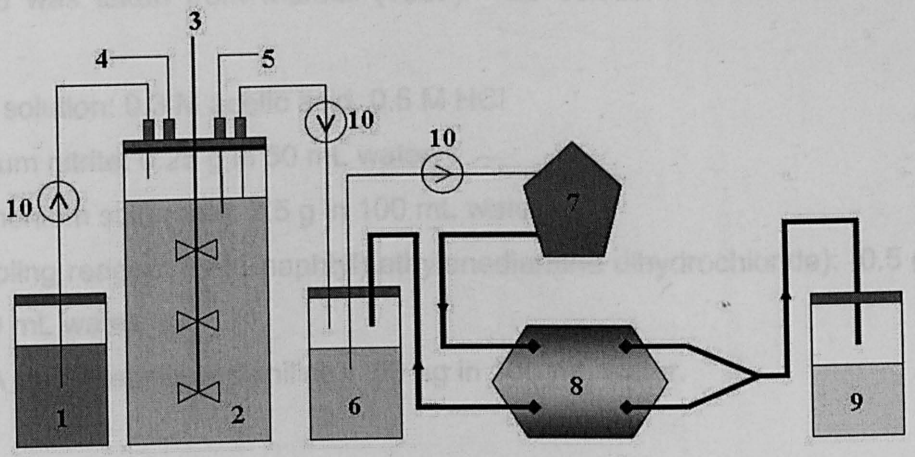
7.5.1 Materials and methods

A larger scale continuous culture system was set up in the laboratory using a 10 L-reactor from LH Fermentation and peristaltic pumps from Watson-Marlow to

operate the system (Fig. 7.10). Since this was a ten-fold scale up of the system based on the 1 L reactor continuous system as described previously (in Fig. 7.1), the flow rate could be expected to be set ten times faster i.e. 10 mL/min. It was found, however, that the maximum flow rate to obtain efficient dye decolourisation was 5 mL/min.

Filtration technology was added to the system to remove residual biomass and particles from the bacterial treatment, hence to improve the quality of the decolourised effluent. The Sartocon ultra-filtration system was composed of mini-cassettes of cellulose triacetate as membrane material (nominal molecular weight cut-off of 10,000 da). The filtration system was operated by an air-driven Sartorius pump, which needed an air compressor (SB-46 Compressor oil 75/50 from Bambi Air Compressors Ltd) to function.

Fig. 7.10 shows the set up of the lab-scale apparatus. The bioreactor was filled with PU foam and with 10 L of nutrient broth (Oxoid) inoculated with a mixed culture of *Pseudomonas* spp. The system was left to stabilise at room temperature (around 25°C at daytime) for 24 hours to allow formation of biofilm on the PU foam.



1 Raw effluent containing 0.2 % starch

2 Bioreactor filled with PU foam and 10 L of bulk liquid

3 Agitator

4 Air vent (with filter)

5 Sampling port

6 Glass container

7 Air-driven pump

8 Micro-filtration system

9 Container (treated effluent)

10 Pumps (flow rate: 5 mL/min)

Figure 7.10 Diagram of the larger scale (10 L reactor) laboratory continuous process.

The feed bottle contained raw effluent received from Stevensons' balance tank. Soluble starch (0.2 %) was added to the effluent and the pH was adjusted to 7.0 using 0.5 M HCl. The system was left to run continuously for fourteen days. The decolourised effluent from the bioreactor accumulated in a container and then passed through the filtration system. The filtered samples were collected through the sampling port every day for analyses. The pH, colour, COD, BOD_5 and toxicity of the samples were measured. The aromatic amines concentration was also determined as described below.

Determination of aromatic amines concentration

The method was taken from Marcali (1957). The solutions needed were as followed:

- Acid solution: 0.3 M acetic acid, 0.5 M HCl
- Sodium nitrite: 0.25 g in 50 mL water,
- Ammonium sulfamate: 2.5 g in 100 mL water,
- Coupling reagent (N-(1-naphtyl) ethylenediamine dihydrochloride): 0.5 g in 50 mL water,
- MDA (4,4'-methylenedianiline): 50 μg in 100 mL water.

A volume of 5 mL acid solution and 2 mL acetone were added to 10 mL sample, as well as 1 mL sodium nitrite and left to stand for ten minutes. A volume of 2 mL of the ammonium sulfamate solution was added, and left for ten further minutes. 2 mL of coupling reagent were then added and the sample left to stand for sixty minutes. Absorbance of samples was then measured at 550 nm using a spectrophotometer.

A calibration curve was obtained using MDA as standard, over the range 0-5 μg/10 mL.

7.5.2 Results

The absorbance *vs.* wavelength scans of the three different effluent samples taken at the three stages of treatment (Fig. 7.11) show an overall decrease in colour absorbance. After treatment with bacteria in the bioreactor, 50-60 % of the colour has been removed. After membrane filtration, a further 30 % of colour was removed, resulting in a final 80 to 90 % colour removal. Fig. 7.12 indicates that there is less than 40 % colour remaining in the reactor after two weeks of continuous experiment. pH values varied between 7.0 and 8.5 over the experimental period.

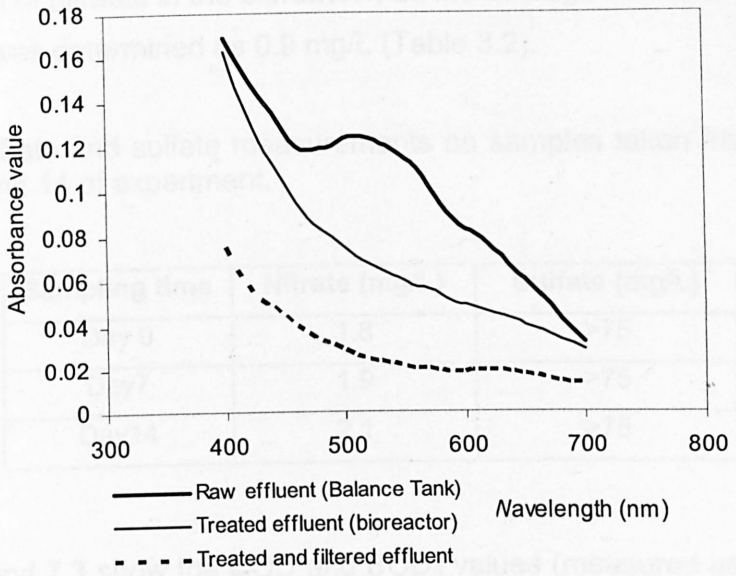


Figure 7.11 Wavelength scans of three effluent samples: raw, treated and membrane-filtered effluent samples.

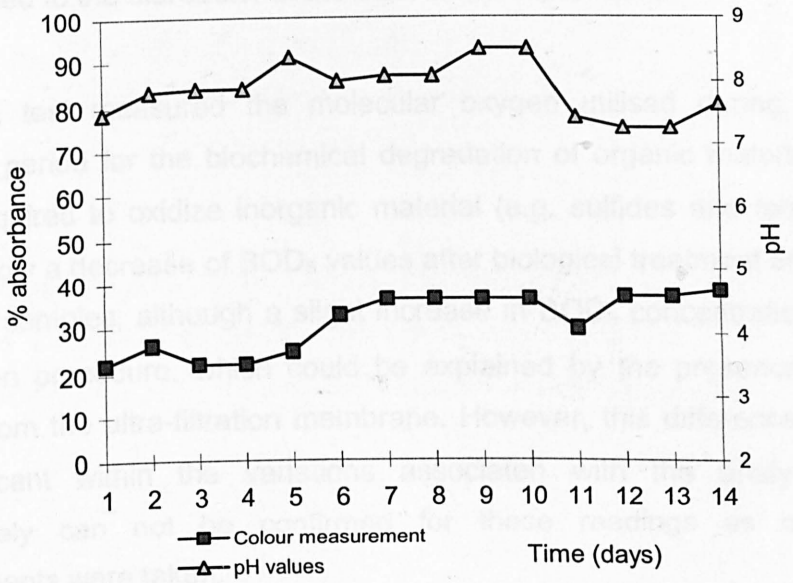


Figure 7.12 Colour and pH measurements over 14 days continuous experiment (samples taken from bioreactor).

Nitrates and sulfates were measured in samples taken from the bioreactor at day 0, 7 and 14 of the experiment (Table 7.1). The results show a higher

concentration of nitrates in the bioreactor, as the average concentration found in raw effluent was determined as 0.9 mg/L (Table 3.2).

Table 7.1 Nitrate and sulfate measurements on samples taken from bioreactor at day 0, 7 and 14 of experiment.

Sampling time	Nitrate (mg/L)	Sulfate (mg/L)
Day 0	1.8	>75
Day7	1.9	>75
Day14	2.1	>75

Tables 7.2 and 7.3 show the COD and BOD_5 values (measured as described in Section 3.3.6), corresponding to chemical and biochemical oxygen demands. The COD values indicate there was a decrease of organic content in the effluent samples after treatment and filtration. The higher value obtained for COD at day 7 compared to day 14 was probably due to the remaining carbon source added to the bioreactor at the start of the experiment.

The BOD_5 test measured the molecular oxygen utilised during five days incubation period for the biochemical degradation of organic material and the oxygen required to oxidize inorganic material (e.g. sulfides and ferrous ions). Results show a decrease of BOD_5 values after biological treatment and filtration of effluent samples, although a slight increase in BOD_5 concentration followed the filtration procedure, which could be explained by the presence of eluted material from the ultra-filtration membrane. However, this difference might not be significant within the variations associated with the analysis, which unfortunately can not be confirmed for these readings as only single measurements were taken.

Table 7.2 COD measurements of effluent samples at different stages of the treatment. The membrane filtered samples were taken at day 7 and 14 (D7 and D14) of the continuous operation after biological treatment.

Sample	COD (mg/L)
Raw effluent + 0.2% starch	3,368
Treated effluent from bioreactor	984
Treated and filtered effluent D7	528
Treated and filtered effluent D14	324

Table 7.3 BOD_5 values for some effluent samples (biologically treated and filtered effluent samples were taken at day 14 of the continuous operation).

Sample	*BOD_5 (mg/L)*
Raw effluent	12.5
Raw effluent + 0.2% starch	18.4
Treated effluent (from bioreactor)	2.5
Treated and filtered effluent	3.4

Toxicity measurements were carried out as described in Section 6.2.2.3. Results are expressed as percentage effect (Fig. 7.13) and reveal that toxicity level decreased after effluent treatment (from around 80 to 20 %).

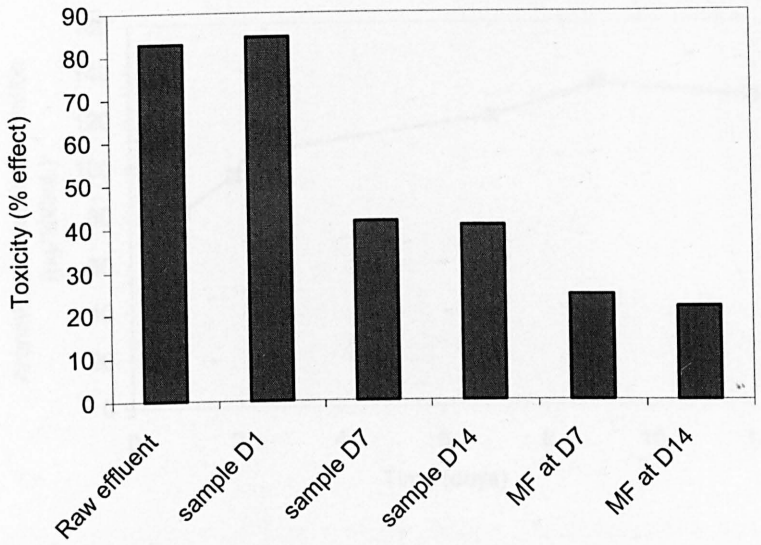


Figure 7.13 Toxicity measurements of 6 different samples (raw effluent, effluent samples taken at day 1, day 7 and day 14 of continuous culture operation, and decolourised effluent samples after membrane filtration at day 7 and 14).

The aromatic amines measured in samples taken form the bioreactor bulk liquid over twelve days of continuous operation are shown in Fig. 7.14. Results of single readings show there was an increase in aromatic amines concentration of approximately 50 µg per 100 mL of sample analysed. This is, however, surprising as an increase in aromatic amines concentration usually leads to an increase in toxicity levels. Instead, bacterial treatment of the effluent caused decrease in toxicity, COD and BOD_5 levels, which shows that biodegradation occurred (Fig. 7.13, Tables 7.2 and 7.3). The increase in aromatic amines concentration may have been the result of bacterial reduction of azo dyes occurring in the bioreactor (within the PU matrix and or in the bulk liquid). The toxicity levels measured might have been caused by aromatic amines as well as other compounds such as residual non-degraded dyes and or other bacterial degradation products.

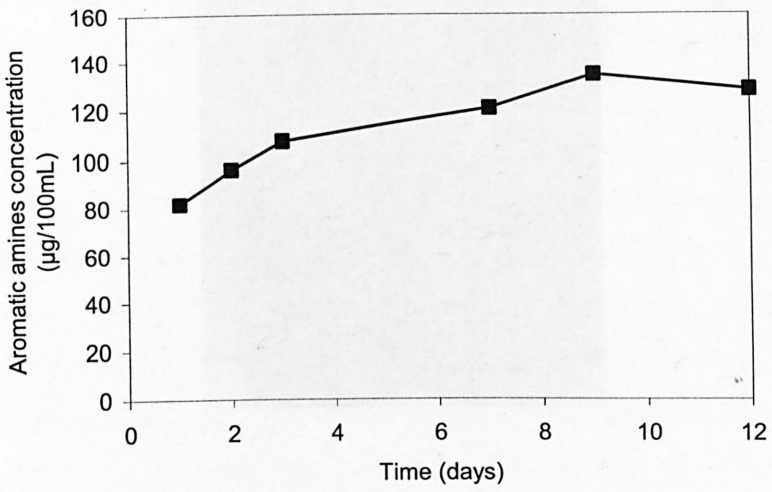


Figure 7.14 Aromatic amines concentration measured over 12 days continuous operation (samples were taken from the bulk liquid within the bioreactor).

The photograph of raw and filtered-treated effluent (Fig. 7.15) illustrates well the decolourisation of the wastewater obtained through the experimental process. The bottle on the left corresponds to raw effluent collected from Stevensons' balance tank. The bottle on the right contained biologically treated and membrane filtered effluent.

Samples of the treated and filtered effluent were collected (around 2 litres) and given to Quantum Clothing Ltd Stevensons for dyeing experiments in order to assess the quality of the water for recycling purposes.

Figure 7.15 Photograph of raw effluent from Stevensons' balance tank (left) and treated-filtered effluent after treatment through the 10 L bioreactor system (right).

7.6 Impact of the developed methods on the potential reuse of water

7.6.1 Materials and methods

Decolourised, filtered effluent samples from the experiments were used to dye cotton fabrics and compare them with those dyed with normal supply water.

The decolourised, filtered effluent samples taken from the lab-scale effluent treatment experiment were used to dye 2 g cotton fabrics in black (with C.I. Reactive Black A), in cranberry pink (with Optisal red 7B) and were also treated with Uvitex DMS (a brightening agent) to assess the whiteness of the fabrics. The dyeing experiments were carried out using direct dyeing procedure with Optisal red 7B, and a standard reactive dyeing procedure with C.I. Reactive Black A. For the assessment of whiteness, as the cotton fabrics were pre-bleached, the samples were incubated with the water samples in the presence of Uvitex DMS at 80°C for 30 minutes only (see Appendix C for the procedures).

Colour of fabrics was assessed using a datamatch system (Datacolor SF600® Plus-CT with the computer software Datacolor Program Shell v3.1, Datacolor International© 1996).

According to Ingamells (1993), three variables (hue, strength and brightness) can be used to describe colour qualitatively. Hue is the attribute of colour (such as red, green, blue, yellow etc), strength corresponds to the amount of colour present, and brightness matches the greyness of a colour. The description of colour is facilitated by the organisation of colour into a three dimensional array using lightness (L), chroma (C) and hue (H), as the coordinates used to describe a colour solid. Other names for chroma are saturation, purity or intensity, but the term chroma is preferred in industry. A quantitative representation of colour can be achieved using three "tristimulus values", i.e. the amount of three matching lights (trichromatic system) required to match the shade considered, and these can be calculated and are designated as X, Y and Z.

The difference in colour or ΔE in CIELAB colour space, between a pair of coloured fabrics is determined by a colour difference formula:

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \qquad (1)$$

Where ΔL*, Δa* and Δb* are correlated respectively with the lightness, chroma and hue difference between the samples.

The results are given as the CIELAB values for the 10° standard observer, illuminant D65 (standard artificial daylight), illuminant A (tungsten light) and the fluorescent illuminant TL84.

The CIE Whiteness index under the 10° illuminant D65 was also measured. It represents whiteness in terms of colorimetric values for the specimen and the chromaticity coordinates of the illuminant.

$$W = Y + 800\,(x_n - x) = 1700\,(y_n - y) \qquad (2)$$

Where x, y and Y are the colorimetric values for the specimen form of illuminant D, and X_n and y_n are the chromaticity coordinates of the light source.

7.6.2 Results

Fig. 7.16 is a photograph of the dyed cotton fabrics using normal supply water and treated-filtered effluent. By visual observations, there is no apparent difference between both sets of samples.

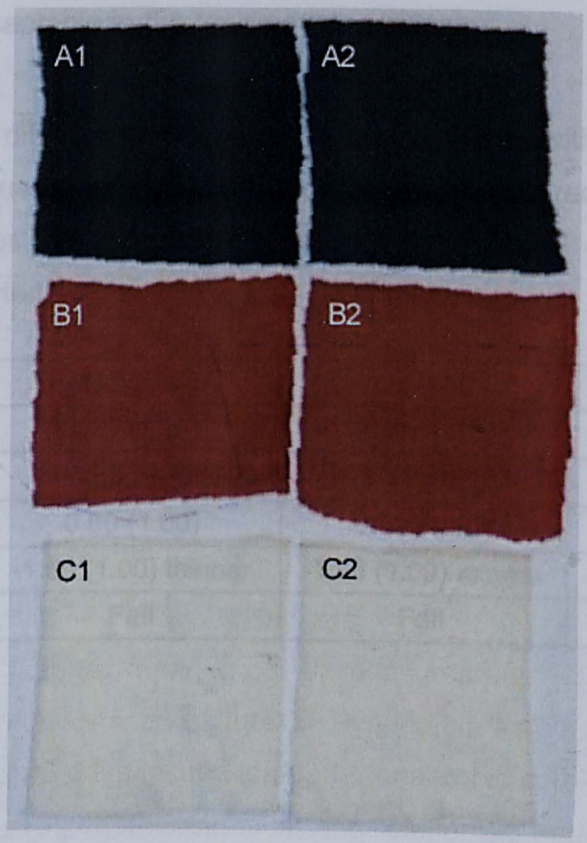


Figure 7.16 Photograph of the cotton fabrics dyed using normal supply water (A1, B1 and C1) and treated-filtered effluent (A2, B2 and C2). The fabric samples A1 and A2 were dyed with Reactive Black A, B1 and B2 with Optisal red 7B, and C1 and C2 were treated with the brightener Uvitex DMS.

Tables 7.4 and 7.5 display the results of instrumental measurements of colour difference between the fabrics dyed using normal supply water and treated-filtered effluent. The ΔE values indicate that there was not much difference between the fabrics dyed in cranberry pink, whereas those dyed in black showed a great difference in shade between them. Even though the instrumental result failed for the black fabric, a textile company such as Stevensons would have found acceptable the difference between both fabrics (A1 and A2) if they were dyed from different batches.

The whiteness index shown in Table 7.6 indicates that there is a negligible difference between the fabrics treated with Uvitex DMS. Such results would be considered as acceptable in the textile dyeing industry.

Table 7.4 Colour difference between cotton fabrics dyed with Reactive Black A using treated effluent and normal supply water, measured under the three illuminants (figures in brackets correspond to standard values where no difference is detected).

	MsD65-10	**MsA-10**	**Ms TL84-10**
ΔE	1.79 (1.50)	2.31 (1.50)	1.69 (1.20)
ΔH* (Hue)	-0.79 (0.75)	-1.71 (0.75)	-0.01 (0.6)
ΔC* (Strength)	0.00 (1.00)	0.00 (1.00)	0.00 (0.8)
ΔL* (Brightness)	-1.60 (1.00) thinner	-1.55 (1.00) thinner	-1.69 (0.80) thinner
Result	**Fail**	**Fail**	**Fail**

Table 7.5 Colour difference between cotton fabrics dyed with Optisal red 7B using treated effluent and normal supply water, measured under the three illuminants (figures in brackets correspond to standard values where no difference is detected).

	MsD65-10	MsA-10	Ms TL84-10
ΔE	0.85 (1.50)	0.93 (1.50)	0.90 (1.50)
ΔH* (Hue)	-0.20 (0.75)	-0.19 (0.75)	-0.27 (0.75)
ΔC* (Strength)	0.01 (1.00)	-0.03 (1.00)	-0.01 (1.00)
ΔL* (Brightness)	-0.83 (1.00) thinner	-0.91 (1.00) thinner	-0.85 (1.00) thinner
Result	**Pass**	**Pass**	**Fail**

Table 7.6 Colour difference between pre-bleached cotton fabrics treated with Uvitex DMS for assessment of the whiteness of the fabrics.

Water used	CIELAB tristimulus values Illuminant D_{65}, 10° standard observer			Whiteness
	X	*Y*	*Z*	
Normal supply water	0.2938	0.3001	89.1	**157.6**
Treated-filtered effluent	0.2977	0.3055	89.4	**145.5**

Overall, the fabrics dyed using recycled water were of similar quality to those dyed with normal supply water. These results (Figs. 7.4 and 7.5) show that effluent treatment used are satisfactory to remove colour and for recycling.

Nevertheless, there is a concern over the amount of salt present in the recycled water as dyeing processes require huge amounts of salt (up to 20 g/L of dyebath during direct dyeing procedures). Consequently, the amount of salt already present in recycled effluent (not removed by biological treatment nor membrane filtration) should be taken into consideration during dyeing processes using the recycled effluent. Therefore, the salt required in the dyeing

bath may be considerably reduced otherwise the quality of dyed fabrics may be affected.

7.7 Suggestions for modifications to the effluent treatment system at Stevensons

Fig. 7.17 shows a simplified diagram of the existing effluent treatment plant at Stevensons. Suggestions for modification to the effluent treatment plant are shown in Figs. 7.18 and 7.19. Both involve the set up of a new biological treatment tank containing the dye-degrading *Pseudomonas* spp. Experiments described in Section 7.5 show that the use of a filtration step in the process can help decrease BOD_5 values, hence improve the efficiency of the process and allow water recycling.

In the system shown in Fig. 7.18, a new biological treatment reactor can be introduced to remove most of the effluent's colour from the balance tank. The following aerobic reactors are kept to help further degradation of the effluent (reduction of COD, BOD_5 and aromatic amines). The micro filtration (0.1 μm) system may be required for water recycling.

However, the integrated aerobic / anaerobic conditions within one reactor tank, provided by the use of a biomass support material (PU foam) with immobilised *Pseudomonas* spp., could be sufficient for treating the effluent. As shown in Fig. 7.19, the main treatment stage consists of the new developed biological treatment system associated with membrane filtration technology. The suggested process could be advantageous as high cost reverse osmosis filtration (50 Å/ 200 M.W.) might not be required for water recycling. Micro (0.1 μm) or ultra (1,000 Å / 500,000 M.W.) filtration might achieve adequate removal of residual biomass and or particles from the biological treatment stage. Therefore, the new system could replace the existing biological treatment tanks and DAF tanks at Stevensons and produce treated effluent acceptable for water recycling.

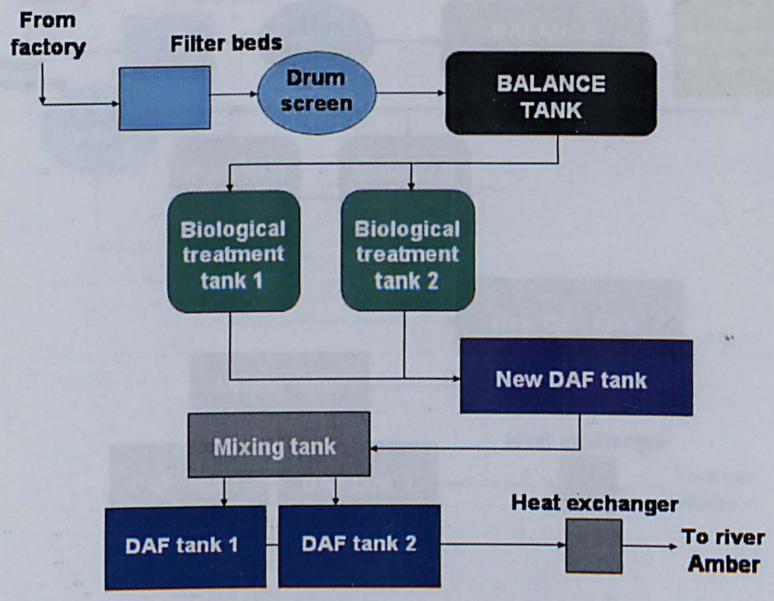


Figure 7.17 Basic diagram of the existing Stevensons' effluent plant

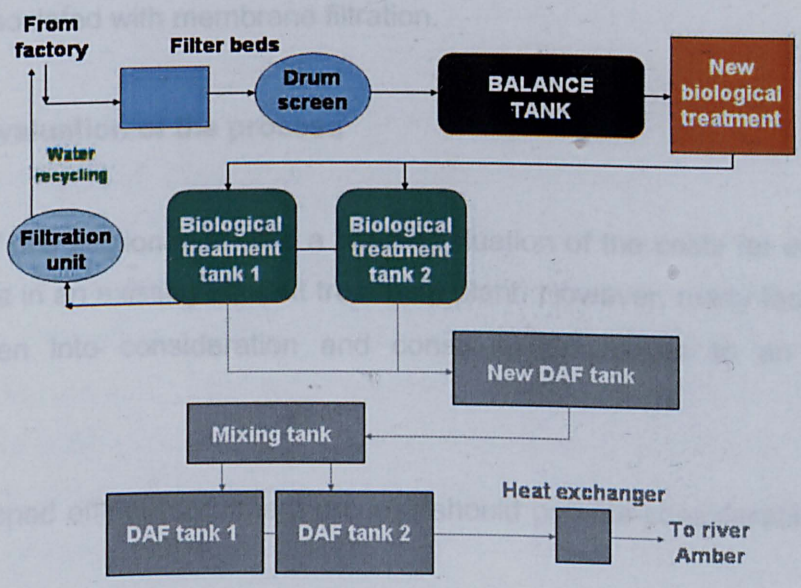


Figure 7.18 Suggestion for Stevensons' effluent plant with an added biological treatment tank and a filtration system for recycling the effluent. The flocculation stage (DAF tanks) and discharge to river have been removed.

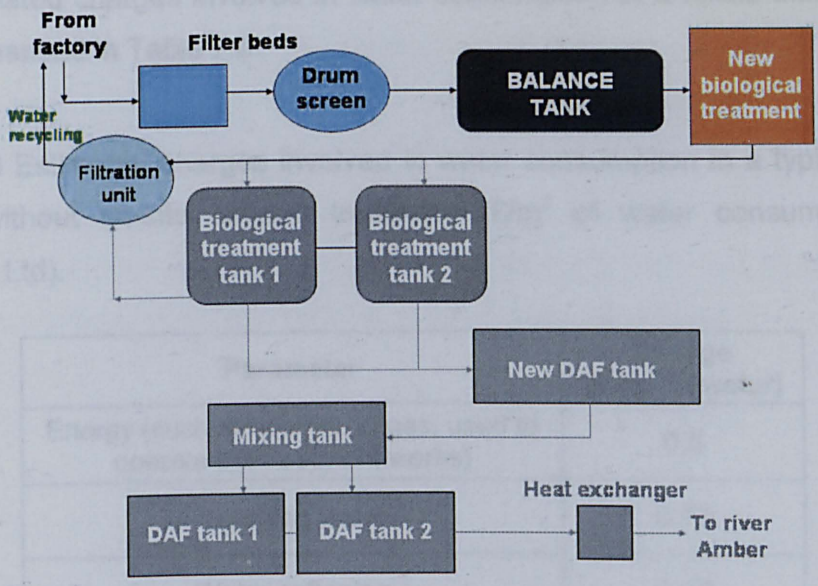


Figure 7.19 Alternative suggestion for the effluent treatment plant at Stevensons with removal of existing biological treatment tanks and DAF tanks. The main treatment stage consists of the new developed biological treatment system associated with membrane filtration.

7.8 Cost evaluation of the process

The aim of this section is to give a basic evaluation of the costs for developing the process in an existing effluent treatment plant. However, many factors need to be taken into consideration and constitute drawbacks to an accurate evaluation.

The developed effluent treatment process should provide considerable savings from:

- Reduced water charges
- Reduced softening costs
- Reduced sewerage charges (through reduction of volume, BOD, suspended solids and colour)
- Lower heat loss (meaning energy savings for the factory)
- Less dependence on water source supplies

The estimated charges involved in water consumption at a textile effluent plant are summarised in Table 7.6.

Table 7.6 Estimated charges involved in water consumption in a typical textile factory without on-site effluent treatment (£/m^3 of water consumed) (MIE Solutions Ltd).

Parameter	Charge (£ /m^3 of water)
Energy (such as electricity, gas, used to operate the treatment works)	0.5
Incoming water	0.75
Water softening	0.08
Water discharge (without treatment)	0.45
Colour surcharge cost (from untreated discharge water)	0.35
Total estimated	**2.13**

The total cost for on-site effluent treatment in a textile effluent plant such as Quantum Clothing Ltd Stevensons is approximately £ 0.6 / m^3.

The estimated total cost per cubic metre of water processed for operating the developed treatment method (appropriately scaled-up to industrial size) can be roughly estimated as £ 0.38 / m^3. This should include the costs for the carbon source (soluble wheat starch 0.2 % w/v) and PU foam if obtained at bulk price. The starch is mainly used to promote and sustain biomass within the biological tank. The amount used could be further reduced if the bacteria are well established on the PU foam and if sludge return is carried out.

Under ideal conditions, the estimated savings made from using the effluent treatment system with recycling of water would remove or considerably reduce charges for incoming water, water softening, water discharge and colour surcharge. The energy cost, nevertheless, might increase due to the added

energy required to run the new treatment system. These estimations do not take into account the cost for installation of the process (new biological treatment tank and filtration unit) which will be negated over the years by the profits made from its use.

Other important factors should also be considered such as filtration costs (replacement filters, electricity for filter pumps) and labour costs.

CHAPTER 8

GENERAL SUMMARY AND DISCUSSION – FURTHER STUDIES

Stringent environmental legislations are constantly re-enforced to stimulate the textile industry to reduce water consumption and reduce the release of pollutants in the environment in order to prevent environmental damages.

Textile effluents need to be treated, not only for colour removal but also for organic pollutants. They contain residual dyes with complex structures and other pollutants such as salts, surfactants, nitrates, metal ions, dispersants, levelling agents, acids and alkali, which make treatment difficult. Numerous techniques for treating effluents are available. One of the conventional treatments for colour removal currently used in many textile companies is chemical flocculation. It is very effective but expensive as it requires the addition of flocculation agent and disposal of large quantities of waste sludge produced. Much research has focused on the use of biotechnology as a cost-effective solution for "end of pipe" treatments. Several microorganisms have been reported to be capable of transforming azo dyes, under certain conditions, into non-coloured products or even completely mineralise them (Chung *et al.*, 1992; Brown *et al.*, 1993; Zissi *et al.*, 1996).

The PhD research was carried out in collaboration with a local dye-house effluent treatment plant (Quantum Clothing Ltd Stevensons), who provided effluent for the study. Basic parameters and ionic composition of the effluent were determined (Chapter 3) and the treatment efficiency was evaluated. It was found that COD and BOD_5 decreased after treatment at Stevensons, ammonia increased in concentration, but most of the metal ion concentrations were not affected by the on-site treatment. Analyses provided useful information on the composition of a typical textile effluent, although the effluent from a treatment plant is unique due to the high variety of dyeing processes. The substances that need to be removed from the textile waste water are mainly COD, BOD_5,

nitrogen, heavy metals and residual dyestuffs, because they are recalcitrant and hazardous to the environment. Their presence in effluents is carefully monitored by the water authorities and failure to remove them from the effluent prior to discharge results in fines.

Studies were carried out to develop methods improving biological treatment of textile effluent. The objectives of the research project involved:

- The biotechnical degradation of dyestuffs in effluents
- The potential combination of biotechnical and membrane technology
- The development of appropriate laboratory / industrial equipment
- The recycling of water for re-use in textile dyeing, and study of its impact on the quality of dyed products

Different strains of *Pseudomonas* spp. have been isolated from Stevensons' activated sludge and were proved capable of decolourising a wide range of selected textile dyes, achieving up to 99 % decolourisation (Section 5.3.1). These bacteria are gram-negative chemoheterotrophic, facultative anaerobes (capable of growing either with or without oxygen). Optimum conditions for dye colour removal were determined by studying the effect of different carbon sources and different polymer supports on decolourisation rates. It was found that the use of soluble wheat starch (0.5 % w/v) as a carbon source improved decolourisation of dye and effluent samples, especially under anaerobic conditions (Section 5.3.2). PU foam was found to be the best bacterial immobilisation material compared to other polymers (Section 5.3.3) and was, therefore, used in experiments to support biomass growth.

A bench-scale continuous culture system was set up using a 1 L-reactor filled with PU foam, which was colonised by a mixed culture of the *Pseudomonas* spp. The efficiency of the system was assessed for effluent treatment. Results from preliminary experiments showed that high percentages of decolourisation were maintained through the three-day continuous operations (Section 7.2.2). The optimum concentration of soluble wheat starch was also determined (0.2 % w/v was used in effluent medium preparations).

Longer periods of continuous operation (up to 14 days) were also carried out using a larger scale bioreactor (10 L). In order to improve the quality of the treated effluent (i.e. removal of residual biomass and particles coming from the reactor bulk liquid), a membrane filtration system was installed following the biological treatment. Resultant treated and filtered effluent samples were used to dye cotton fabrics. The colour difference between fabrics dyed using normal supply water and effluent water was analysed. Results showed that the effluent treatment is satisfactory for colour removal and re-use in dyeing processes (Section 7.6).

The decolourisation of dye samples using laccase was carried out and compared to the bacterial decolourisation of dye and effluent samples. Results indicated that the enzymes only partially decolourised four dyes, although it is important to note that the experiments were carried out without mediators, which could have increased decolourisation rates. In relation to the developed continuous culture system for effluent treatment, the use of enzymes is not advantageous for treating the mixed effluent containing a wide range of dyes. Nevertheless, their use in textile dyebaths might be best suited and give more efficient results, especially when immobilised. The introduction of enzymatic dye degradation in dyebaths as pre-treatment could lead to further improvement of the effluent treatment system.

Many virtues can be perceived from the bio-treatment system developed through the research. The formation of integrated anaerobic/aerobic conditions within a single vessel is very advantageous. PU foam, a porous polymer matrix is robust to microbial degradation and is water insoluble. The colonisation of the foam by bacteria creates anoxic zones within its honeycombed structure. Ideally azo dyes can be reductively cleaved under anoxic conditions within the pores of the PU matrix, and biodegradation of the aromatic amines arising from the azo bond cleavage occurs in the aerobic zones, within and outside the foam (in the bulk liquid of the reactor). The use of such integrated system has been reported by Tan *et al.* (1999), who believe it to be a good strategy for complete

mineralisation of azo dyes, provided the presence of a co-substrate in the system for the formation of anaerobic micro-niches.

Soluble wheat starch (0.2 % w/v) was successfully used as a carbon source during laboratory-scale experiments (Chapters 5 and 7). Its presence in the reactor was required to support biomass growth. Controls indicated that the absence of carbon source in dye or effluent samples showed decrease or lack of decolourisation. Therefore, the metabolism of dye degradation may either be linked to the presence of a carbon source or to biomass concentration. Carliell *et al.* (1996) stated that a carbon source was essential for decolourisation to occur. Many researchers believe that high decolourisation rates are also linked to low redox potentials (Beydilli *et al.*, 1998; Carliell *et al.*, 1995). However, this statement was also refuted by others (Chinwetkitvanich *et al.*, 1999). Further efforts would, therefore, be valuable to better understand the mechanisms involved in dye degradation.

The production of sludge can be considerably reduced through the use of the bio-treatment system, and ease the problem of sludge disposal. As suggested in Section 7.7, many existing treatment tanks (DAF and biological) may be removed after modifications to the effluent treatment plant and the use of a reduced number of reactors/tanks would contribute to the reduction of sludge production.

The combination of other techniques such as membrane filtration with biotechnology was also found to be advantageous to the efficiency of the treatment system (decrease in toxicity levels and BOD_5) and make water recycling possible. Indeed, the research demonstrated the feasibility of re-using the treated textile effluent in dyeing processes without impact on the quality of dyed cotton fabrics. This could consequently save water resources. The bio-treatment and filtration system might be successfully used in industry as an improvement of existing effluent treatment plants. In such prospects, considerable savings could be made by textile companies applying the

suggested modifications to their effluent plant as complete re-structure is not required.

There are, however, many limitations to the bio-treatment and filtration system that need to be considered. The stability of the proposed set-up in term of biomass is not known and certainly needs further study. As stated in Section 7.2.2, the continuous culture experiments were not carried out in rigorous sterile conditions. The *Pseudomonas* strains can not, therefore, be expected to remain dominant within the reactor, especially over a long period of continuous operation, with non-sterile effluent brought into the system for treatment. The presence of carbon source (starch) and the high organic contents of the effluent (high BOD_5) contribute to the rapid development of a large microbial community mainly composed of the selected *Pseudomonas* spp., under ideal conditions, but also composed of other bacteria and or fungi within the reactor, which functions as a small ecosystem. In any ecosystem, selecting pressure is exercised / put on the microorganisms, which are, therefore, competing between each other for survival. It is possible that, over a long period of time, the dye decolourising bacterial strains might be taken over by other bacterial strains and disappear from the reactor. This problem should be resolved through the use of PU foam, provided that the *Pseudomonas* spp. are robustly established on the polymer matrix as a biofilm, and that the presence of a carbon source can sustain them for re-population of the reactor bulk liquid.

Cases of polyurethane degradation by some bacterial strains have been reported. Some early investigations by Osserfort *et al.* (1966) have nevertheless established that PU for example can be biodegradable. Some microbial degradation of PU coatings by biofilms formed on surfaces such as prostheses or implants in the medical environment have also been reported (Costerton *et al.*, 1995; Marshall, 1992). According to Albertsson *et al.* (1987), UV light or oxidizing agents (e.g. UV sensitiser) can enhance biodegradation of PE, and without them the natural biodegradation process would take more than ten years (Albertsson *et al*, 1988). Regular replacement of PU foam in the system would, therefore, be required. Pragmatic alterations would have to be carried

out if the suggested modifications are made to an effluent treatment plant. For example, the incorporation of PU foam in the biological treatment tanks would be required to develop the integrated anaerobic/aerobic condition and thus improve decolourisation. Further experimentations need to be carried out to study the conditions of operation (e.g. aeration might still be required in the tanks).

There is another concern arising from the use of a carbon source in the process to ensure that there is sufficient biomass density in the system for effluent decolourisation. The residual amount of carbon source present in the recycled effluent might contribute to BOD_5 and give rise to microbial growth in dyebaths that may interfere with the safety of company staff and with the dyeing processes. Fortunately, since those are mainly carried out at high temperature (over 80°C), the proliferation of microbes is very unlikely.

The repeated recycling of waste water could lead to possible accumulation of micro contaminants, which could affect the stability of the system and decrease the quality of dyeing processes. The presence of high concentration of salts can also be problematic, although dyeing procedures require high amounts of salts and a reduction in the quantity of salt added to dyebaths could be the solution. It has to be noted that a superior treated effluent quality is not always required for water re-use within the company if it is used for equipment cleaning and water cooling systems.

The implementation of a filtration unit at an effluent treatment plant such as Stevensons is not essential to the proposed treatment process. It was initially suggested because the removal of residual biomass and particles from the treated effluent was required for re-use of the effluent in dyeing experiments. Indeed, the existing processes at Stevensons (DAF, flocculation and sedimentation) are very efficient. The installation and use of a filtration unit would be very expensive (operation costs, maintenance, filter replacements, labour costs etc) and, therefore, unnecessary.

Intense similar research has been carried out to improve industrial waste water using immobilised microbes for bioremediation of pollutants (Na *et al.*, 2000; Silva *et al.*, 2002 and van Groenestijn *et al.*, 2002). Buitron *et al.* (2004) have demonstrated the mineralisation of an azo dye using aerobic bacteria immobilised on a porous support. Investigations for improving waste water using combined technologies such as filtration can be found in recent publications (Jaap *et al.*, 2000; Nyström *et al.*, 2003 and Jhawar *et al.*, 2003).

Further studies evolving from this research project should include advanced optimisation of the process and integration of different technologies. Proper tests on an industrial scale are essential to validate the efficiency of the treatment process. More studies on the effect of laccase and mediators in decolourisation of dyebaths would be interesting and may provide cost-effective improvement of textile effluent treatments.

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APPENDIX A

ENVIRONMENT AGENCY
Reference: PAUL REEVES EXT 2222
3rd July 2000

COATS VIYELLA CLOTHING STEVENSON
COATS VIYELLA. CLOTHING
STEVENSONS
AMBER DYE WORKS , AMBERGATE
BELPER, DERBYSHIRE
DE56 2EX

For attention: MR RAY SMITH

Site no: **50896300 AMBER DYEWORKS, WASTE TREATMENT** PLANT, TE
Purpose : CA Mechanism: S
Sampled By: S856

Dear Sirs

Reported below are the results, measured and analysed, for determinands in your Consent to Discharge with absolute limits (or no limits), taken at 10.55 hours on 7 Jun 2000.

Determinand		Unit		Reading
Ammonia -	**As N**	**mg/1**		**2.2**
Arsenic -	As As	ug/1	<	1
Bod 5 Day	0.5 Atu	mg/1	<	2.85
Cadmium	As Cd	ug/1	<	.1
Chromium	**As Cr**	**ug/1**		**7**
Copper -	**As Cu**	**ug/1**		**1.97**
Lead -	As Pb	ug/1		.441
Nickel -	As Ni	ug/1	<	5
Optical Density	At 400nm (Filtered Samp	ABS/cm		.021
Optical Density	At 450nm (Filtered Sam	ABS/cm		.028
Optical Density	At 500nm (Filtered Sam	ABS/cm		.023
Optical Density	At 550nm (Filtered Sam	ABS/cm		.007
Optical Density	At 600nm (Filtered Sam	ABS/cm		.004
Optical Density	At 650nm (Filtered Sam	ABS/cm		.002
Optical Density	At 700nm (Filtered Sam	ABS/cm		.001
Ph -	As Ph Units	PHUNIT		7.7
Solids Suspended @105C		mg/1		14
Temperature Water		CEL		20
Zinc -	**As Zn**	**ug/1**		**27.3**
Optical Density	At 490nm (Filtered Samp	ABS/cm		.027
Cod -	**As O_**	**mg/1**		**80**
Nitrogen Total Oxidized	As N mg/1	<1		
Chloride Ion - As Cl		mg/1		1240
Carbon organic Dissolved	As C	mg/1		15.3
4-Chloro-2-Methylphenol	{P-Chloro-O-Cre	ug/1	<	.1
4-Chloro-3-Methylphenol	{P-Chloro-M-Cre	ug/1	<	.1
2,4-Dimethylphenol		ug/1	<	.1
3,5-Dimethylphenol		ug/1	<	.1
4-Methylphenol {P-Cresol}		ug/1	<	.1

The Environment Agency

Lower Trent Area, Trentside Offices, Scarrington Road, West Bridgford, Nottingham NG2 5FA. Tel 01159 4-55722 Fax 01159 817743

Determinand	Unit		Reading
3-Methylphenol {M-Cresol}	ug/1	<	.1
2-Methylphenol {O-Cresol}	ug/1	<	.1
Alkalinity Ph 4.5 - As Caco3	mg/1		379
Detergents Anionic Synthetic - As M.Ot	mg/1		.313
Metals Total (Cr, Ni, Pb, Zn) (Grp 45)	**ug/1**		**39.7**
2,5-Dichlorophenol	ug/1	<	.1
2,5-Dimethyl Phenol	ug/1	<	.1
2,3-Dimethylphenol	ug/1	<	.1
2,3-Dichlorophenol	ug/1	<	.1
2-Ethylphenol	ug/1	<	.1
Optical Density At 800 Nm (Filtered Sam	ABS/cm		.001
Optical Density At 750 Nm (Filtered Sam	ABS/cm		0
Phenol	ug/1		4.7
Detergents Non-Ionic - As Symperonic Np	mg/1		.08
Optical Density At 420nm (Filtered Samp	ABS/cm		.022
Optical Density At 410nm (Filtered Sam	ABS/cm		.022
Optical Density At 430nm (Filtered Samp	ABS/cm		.023
Optical Density At 440nm (Filtered Sam	ABS/cm		.026
Optical Density At 460nm (Filtered Sam	ABS/cm		.03
Optical Density At 470nm (Filtered Sam	ABS/cm		.03
Optical Density At 480nm (Filtered Sam	ABS/cm		.029
Optical Density At 510nm (Filtered Sam	ABS/cm		.018
Optical Density At 520nm (Filtered Sam	ABS/cm		.013
Optical De sity At 530nm (Filtered Sam	ABS/cm		.01
Optical Density At 540nm (Filtered Samp	ABS/cm		.008
Optical Density At 560nm (Filtered Sam	ABS/cm		.006
Optical Density At 570nm (Filtered Samp	ABS/cm		C05
Optical Density At 580nm (Filtered Sam	ABS/cm		.005
Optical Density At 590nm (Filtered Sam	ABS/cm		.004
Optical Density At 610nm (Filtered Sam	ABS/cm		.004
Optical Density At 620nm (Filtered Sam	ABS/cm		.003
Optical Density At 630nm (Filtered Sam	ABS/cm		.003
Optical Density At 640nm (Filtered Sam	ABS/cm		.003
Optical Density At 660nm (Filtered Sam	ABS/cm		.002
Optical Density At 670nm (Filtered Sam	ABS/cm		.002
Optical Density At 680nm (Filtered Sam	ABS/cm		.002
Optical Density At 690nm (Filtered Sam	ABS/cm		.001
2-Chlorophenol	ug/1	<	.1
2,4-Dichlorophenol	ug/1	<	.1
2,4,5-Trichl orophenol	ug/1	<	.1
2,4,6-Trichlorophenol	ug/1		.137

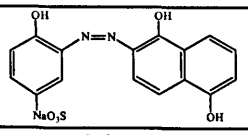
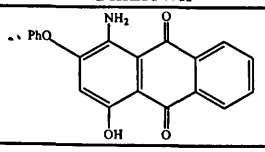
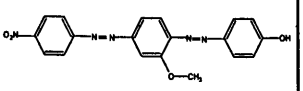
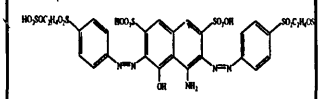
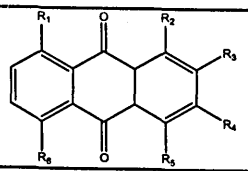
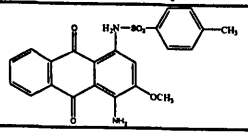
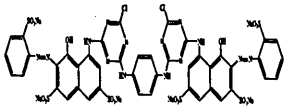
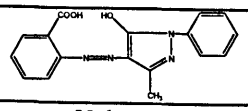
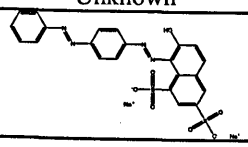
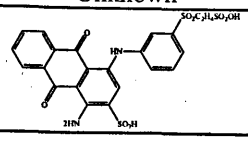
If you have any queries about any of these results, please do not hesitate to contact me at this office.

Yours faithfully

JEFF DOLBY
Environment Protection Manager (Area)

APPENDIX B

Table 1 List of dyes with their wavelength at λmax and chemical structure.

Sample	*Dye (commercial name)*	*Colour Index (C.I.)*	*Wavelength (nm)*	*Chemical Structure*
1	Diamond black PV200	Mordant Black 9	530	
2	Diamond black PLC	Mordant Black 8	530	Unknown
3	Dianix red EFB	Disperse Red 60	590	
4	Dianix goldbrown	Disperse orange 29	450	
5	Remazol black B	Reactive Black 5	580	
6	Lanaset blue 5G	-	610	
7	Terasil rosa 2GLA	Disperse Red 86	530	
8	Evercion red E4B	Reactive Red 120	510	
9	Irgalan gold 2GL	Acid Yellow 59	400	
10	Levafix red E4BA	Reactive Red 158	510	Unknown
11	Levafix yellow gold E-G	Reactive Yellow 27	430	Unknown
12	Acid Red 73	Acid Red 73	520	
13	Neutrilan Black	Acid Black 194	600	Unknown
14	Everzol blue R special	Reactive Blue 19	600	

APPENDIX C

DYEING PROCEDURES

QUANTUM CLOTHING STEVENSONS Recipe Print Date, 16110103 Time 14:31:26 Page 1

Recipe 0071 C 1001 VLWO133PD **REACTIVE BLACK A**
Customer 0071 PENNANT CLOTHING LTD K

Quality VLWO133PD LADIES FASH A/HOLE & SHDR S/WAIST L/SLV

Bath 01 Quantity
INJECT BRINE TO THE EQUIVALENT OF (100 G/L SALT)
SACO COMMON SALT (AS BRINE) 100.0000 G/L
FILL HOT TO LEVEL 2 - INJECT TANK
DP5535 MILLSCOUR SDC (DP5535) 3.0000 G/L
---End Prep
RAISE TO 50.C - RUN 10' - CHECK pH (6.5-7.5)
RAISE TO 60.C -- RUN S'
INJECT DYES (TOP UP TO LEVEL 3)
CHECK SG CORRECT WITH BAGGED SALT ON pH STEP
RBA REMAZOL BLACK A 10.7600%
---End Prep
RUN 30' AT 60.C - COOL TO 40.C
INJECT DOSE TANK OVER 60'
CA CAUSTIC liq - DILUTE WELL 2.0000 G/L
---End Prep
RAISE TO 50.C - CHECK pH AND RECORD---------->
RUN 60' AT 50.C - SHOW
RINSE 5' COLD - LOW SPEED HYDRO 2'
RINSE 5' AT 70.C
***FOR ADDS - COOL TO 40.C - ADD DYE - RUN 15'
RAISE TO 60.C - RUN 20' – SHOW****
FILL HOT TO LEVEL 3 - INJECT TANK
AC ACETIC ACID 1.0000 G/L
---End
Prep
RAISE TO BOIL - BOIL 10'
DROP - RINSE HOT 3'- DROP
FILL HOT LEVEL 3 - RAISE TO BOIL - BOIL 10'
DIRECT COOL TO 30.C - DROP
LOW SPEED HYDRO 2'
FILL MIXED TO LEVEL 3 - INJECT TANK
SUCFC SUPERFIX CFC 1.0000%
---End
Prep
RUN 10'- INJECT TANK

CEPNP CERENINE PNP 3.0000%

Recipe	0071 C 1001 VLWO133PD		**REACTIVE BLACK A**
Customer	0071	PENNANT CLOTHING LTD	K
Quality	VLWO133PD	LADIES FASH A/HOLE & SHDR S/WAIST L/SLV	
Quantity			

----------End Prep

RUN 10' AT 3O.C -- SHOW (DYER CHECK SHADE AND HANDLE)
WHEN OK - DROP
HYDRO AND TUMBLE - UNLOAD INTO TUBS WITH SEPARATORS

Recipe 0148 V 13838/OB ASSORTED **CRANBERRY (OPTISAL RED 73)**

Customer 0148 GHOST LIMITED W

Quality ASSORTED

Bath 01 Quantity

OP NOTE: -GARMENTS MUST BE SHAKEN OUT AND LOADED INDIVIDUALLY

FILL COLD TO LEVEL '5' (centre of drum) - INJECT TANK

SUDG	SUPERLUBE DG	1.0000 G / L
WEAPW	WETTER APW	0.5000 G / L

---End Prep

RAISE TO 25.C - RUN 45' - DROP
FILL COLD TO LEVEL '5' (centre of drum)
RAISE TO 25.C - RUN 135' -- DROP
FILL HOT TO LEVEL 3 - INJECT TANK

MIXAN	MILLSCOUR XAN	2.0000 G / L

---End Prep

RAISE TO 60C - RUN 20' - DROP
RINSE MIXED 5' - DROP
FILL COLD TO LEVEL 2 - INJECT TANK

SUDG	SUPERLUBE DG	1.0000 G / L
WEAPW	WETTER APW	0.5000 G / L
APBC	APOLLENE BC 120	0.3000 G / L

---End Prep

ADD VIA MANUAL TANK USING MIXER

SACOM	COMMON SALT (BAGGED SALT)	4.0000 G / L

RUN 5'
DOSE COLOUR OVER 10'

DBFGLE	SOLOPHENYL BLUE FGLE 220%	0.0069%
DOR7B	OPTISAL RED 7B	2.2000%
DYSF2RL	INDOSOL YELLOW SF2RL	1.2800%

---End Prep

RAISE TO 95.C (2.C/min) - RUN 30' - SHOW
****
**********SEE DYER BEFORE WEIGHING SALT**********
****
DOSE VIA MANUAL TANK OVER 45' (70% PROGRESSION)

SACOM	COMMON SALT (BAGGED SALT)	8.0000 G / L

RUN 15' AT 95.C
DOSE VIA MANUAL TANK OVER 30'

Recipe 0148 V 13838/OB ASSORTED **CRANBERRY (OPTISAL RED 7B)**

Customer 0148 GHOST LIMITED W

Quality ASSORTED

		Quantity
SACOM	COMMON SALT (BAGGED SALT)	8.0000 G/ L

RUN 45' AT 95.C
INDIRECT COOL TO 70.C - SHOW

WHEN OK FOR SHADE ------ DROP BATH
FILL MIXED LV3 RUN 3' - DROP -----FILL COLD LV3 RUN 3' - DROP

FILL MIXED TO LEVEL 3 - ADD VIA MANUAL TANK

OPF	OPTIFIX F	2.0000%

RAISE TO 50.C, RUN 10'- INJECT

CA	CAUSTIC liq - DILUTE WELL	2.0000%

-------End Prep

RUN 10'- DROP
RINSE MIXED 3' – DROP ---------- RINSE MIXED 3' - DROP

FILL MIXED TO LEVEL 3 - INJECT TANK

CEPNP	CERENINE PNP	0.4000%

-------End Prep

RUN 10' AT 30.C -- SHOW (DYER CHECK SHADE AND HANDLE)
WHEN OK -----DROP
HYDRO AND TUMBLE - UNLOAD INTO TUBS WITH SEPARATORS

Recipe 0148 C 50000 COTTON **WHITE (UVITEX DMS)**
Customer 0148 GHOST LIMITED W

Quality COTTON ASSORTED

Quantity

Bath 01

FILL HOT TO LEVEL 2 - INJECT TANK

WEAPW WETTER APW 1.0000 G/L

-------End Prep
RUN C- INJECT
ODMSX UVITEX DMS-X 0.3500%

-------End Prep

RAISE TO 80.C - INJECT
TKPP T.K.P.P. 50% liq 2.5000 G/L

-------End Prep
PREPARE MANUAL TANK - DOSE OVER 10'
HS HYDROS 2.0000 G/L
RAISE TO BOIL - BOIL 30' -- SHOW
DIRECT COOL OVER 10' - DROP
FILL MIXED TO LEVEL 3 - INJECT TANK
CEPNP CERENINE PNP 2.0000%

-------End Prep
RUN 10'AT 30.C - DROP
HYDRO AND TUMBLE - UNLOAD INTO TUBS WITH SEPARATORS